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DNA Vaccination against HuD Antigen Elicits Antitumor Activity in a Small-Cell Lung Cancer Murine Model

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Abstract

There is a clinically significant correlation between the presence of an antibody against the paraneoplastic encephalomyelitis antigen HuD and the limitation of tumor spread in patients with small-cell lung cancer (SCLC). This suggests that HuD is a possible target molecule for antitumor immunotherapy against SCLC. We have hypothesized that anti-HuD immunity suppresses *in vivo* growth of HuD-expressing tumor cells. In this study, Colon 26, a murine adenocarcinoma cell line, stably transfected with the HuD gene (Colon 26/HuD cell) was used as a target cell, and the immunity against HuD was evoked by intramuscular injection of a HuD-expressing plasmid, a technique of DNA vaccination previously used in BALB/c mice. Colon 26/HuD cells were injected subcutaneously and tumor size was calculated as a product of width and length. Antitumor activity was investigated by using two different lots of Colon26/HuD cells in two protocols: Protocol 1, in which either Colon 26/HuD or Colon 26 cells were injected in each side, and Protocol 2, in which Colon 26/HuD cells alone were injected. The size of Colon 26/HuD tumors obtained from mice vaccinated with HuD-expressing plasmid was significantly smaller than those from negative control plasmid-vaccinated mice (86.6 ± 29.9 versus 195.3 ± 48.1 mm^2, $P < 0.05$ in Protocol 1; 107.7 ± 12.8 versus 156.6 ± 22.8 mm^2, $P < 0.05$ in Protocol 2). Moreover, the *de novo* DNA synthesis of spleen cells obtained from HuD-vaccinated mice was significantly enhanced. In addition, anti-HuD antibody was found in individual sera obtained from HuD-vaccinated mice. DNA vaccination with mouse HuD antigen suppressed HuD-expressing tumor growth in a murine SCLC model.

Top
Abstract
Introduction
Materials and Methods
Results
Discussion
References

Introduction

Paraneoplastic neurologic syndromes are disorders of the nervous system that occur in association with cancer but are not a direct result of either primary or secondary lesions within the brain itself (1). Paraneoplastic encephalomyelitis (PEM) and/or paraneoplastic sensory neuronopathy (PSN) is one such disorder sometimes associated with small-cell lung cancer (SCLC) (2). Pathologically, the disease is characterized by neuronal loss, gliosis, and inflammatory infiltrates of the neuraxis, indicative of an immunologic pathogenesis. HuD antigen is a human neuronal RNA-binding protein and a homologue of the drosophila protein embryonic lethal abnormal visual system (*Elav*) (3). Patients with PEM/PSN and SCLC develop an intense immune response against HuD antigen or its homologous Hu antigens, HuC and Hel-N1, which are expressed in the nuclei of tumors and neurons of the peripheral and central nervous systems (2). This immune response is characterized by the presence of anti-Hu antibodies in serum and cerebrospinal fluid and deposits of anti-Hu immunoglobulin (Ig)G within the nervous system and tumor itself (2, 9, 10). The mechanism by which this immunoresponse to Hu antigens occurs in PEM/PSN is largely unknown. However, since HuD is the only Hu antigen expressed in SCLC tumors, it would appear to play a central role in triggering an anti-Hu immune response in SCLC (5, 11, 12). More importantly, the presence of anti-HuD antibodies in sera of patients with SCLC is directly related to the limitation of tumor spread. Approximately 15% of patients who have SCLC without neurologic dysfunction have anti-Hu antibodies in their sera. Most of these patients' tumors are confined to the thorax at diagnosis (13). In contrast, more than 50% of patients who have SCLC without anti-Hu antibodies have metastatic disease at the time of diagnosis (2). Clinical reports of spontaneous SCLC tumor remission in patients with PEM/ PSN and anti-Hu antibodies (14) suggest that the activation of anti-HuD immunity leads to a more favorable prognosis.

The effect of DNA vaccine against cancer and various infections (such as influenza) has recently been investigated. In these studies, it has been demonstrated that direct injection of a plasmid coding for carcinoembryonic antigen (15) or viral proteins (16) produces protective antibodies and elicits a cell-mediated immune response. Compared with orthodox vaccines consisting of tumor proteins or viral components, DNA vaccination allows evaluation of host immunity against transgene-encoding protein without any process of protein purification.

The function and distribution of HuD in the murine nervous system are similar to that in humans. We have therefore hypothesized that DNA vaccination with HuD- expressing plasmid will induce antitumor immunity against HuD-expressing tumors in a murine model. Because there is no available murine SCLC cell line, we have used a murine adenocarcinoma cell line with stable expression of mouse HuD antigen as an SCLC tumor model in this study.

▶ Materials and Methods

Construction of the Plasmid

The full-length sequence (1,158 base pairs [bp]) of mouse HuD antigen (mHuD) was amplified by polymerase chain reaction using complentary DNA (cDNA) obtained from the brains of BALB/c mice (17). Unique *Sma*I restriction sites were added to both ends of the mHuD sequence by using the following primers: 5'-TCCCCCGGGTCAAAGATGGAGTGGAATGGCTTGAAG-3' and 5'-TCCCCCGGGTCAGGATTTGTGGGCTTTGTTGGTTTT-3'. After treatment with *Sma*I, the mHuD sequence was subcloned into the multiple cloning site of the mammalian cell expression plasmid pCI-neo Vector (Promega, Madison, WI) driven by the cytomegalovirus (CMV) major immediate/ early promoter/enhancer. It generated a plasmid with a right-oriented mHuD sequence, designated pCMV.mHuD(+), and a plasmid with the mHuD sequence in the opposite orientation, pCMV.mHuD(−), which was used as a negative control plasmid. Preparation of the plasmids was performed using a plasmid purification kit (Qiagen Plasmid Mega kit; Qiagen, Inc., Valencia, CA). The nucleotide sequence of mHuD in pCMV.mHuD was determined by dye terminator cycle sequencing and was identical with the alternatively spliced short form of mHuD. A 42-bp fragment was deleted from mHuD at the same site, as reported in rat and human HuD sequences (2, 18).

Mice and Cell Lines

Female 5-wk-old BALB/c mice (H-2^d), purchased from Charles River Japan (Yokohama, Japan), were used throughout this study. Mouse fibroblasts NIH/3T3 (ATCC CRL-1658) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc., Rockville, MD), and 10% heat-inactivated calf serum at 37°C in 5% CO_2 (19). Mouse adenocarcinoma Colon 26 cells (H-2^d) were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin G, and 50 μg/ml streptomycin at 37°C in 5% CO_2. Because SCLC cell lines from BALB/c mice were not available, mHuD stable transfectants (Colon 26/HuD) were prepared by electroporation (290 V, 625 mF, 7.70 ms) of 10 μg of pCMV.mHuD(+) into parent Colon 26 cells, and selected in medium containing 800 μg/ml of G418 sulfate (Geneticin; Life Technologies). Several lots of transfectants (Colon 26/HuD cells) were grown separately in two subculture generations and frozen. The same passages of Colon 26/ HuD and Colon 26 cells were used in the experiments.

Northern Blot Analysis

To evaluate mouse HuD antigen expression *in vitro*, we transfected 10 μg of either the (+) or (−) isomer of plasmid pCMV.mHuD into NIH/3T3 cells by electroporation (290 V, 625 mF, 6.86 ms). After 72 h incubation, total RNA was extracted as previously described (20). Ten micrograms of total RNA was electrophoresed in 0.8% agarose gel containing 1.6 M formaldehyde, 1× 50 mM 3-(*N*-morpholino)propanesulfonic acid, and 10 mM ethylenediaminetetraacetic acid (EDTA), and transferred to a Hibond N nylon membrane (Amersham, Arlington Heights, IL). The membrane was hybridized with [^{32}P]-labeled HuD cDNA probe, washed at high stringency, and exposed to X-ray film for 3 h at room temperature. After probing with mHuD cDNA, the membrane was stripped and probed

with [^{32}P]-labeled γ-actin as a control (20).

Western Blot Analysis

NIH/3T3 cells transfected with 10 μg of HuD plasmid by electroporation were harvested after 72 h incubation. Mouse HuD stable transfectant (Colon 26/HuD) or parent Colon 26 cells were also harvested by treatment with trypsin-EDTA and washed with phosphate-buffered saline (PBS). These harvested cells were lysed with a solution (150 μl per 10-cm culture dish) containing 1 mM (2S,3S)-trans-epoxysuccinyl-L - leucylamido - 3 - methylbutane (Sigma, St. Louis, MO), 1 mM phenylmethylsulfonyl fluoride (Sigma), 0.5% Nonidet P-40, 100 mM NaCl, and 50 mM phosphate buffer (pH 7.4). After centrifugation at 18,000 × *g* for 5 min at 4°C, the solubilized protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7% polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-PSQ; Millipore, Bedford, MA). The blots were blocked in BlockAce (Dai-nippon Pharmaceutial Co., Ltd., Tokyo, Japan) containing 0.1 mg/ml bovine serum albumin (Fraction V; Sigma). The initial antibody was obtained from diluted serum (1:5,000) of a patient with PEM diagnosed at the Department of Neurology, Juntendo University Hospital (Tokyo, Japan). Immunohistochemical staining demonstrated that the patient's serum reacted with mouse cerebral tissue (21). In addition, Western blot analysis demonstrated that the patient's serum reacted with a 41-kD HuD band in the cell lysate of BALB/c mouse brain (*see* Figure 1b). After incubation for 16 h, the blots were probed with peroxidase-conjugated goat antihuman IgA + IgG + IgM (H+L) (1:5,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at 25°C. The blots were developed with the ECL Western blotting system (Amersham International PLC, Buckinghamshire, UK).

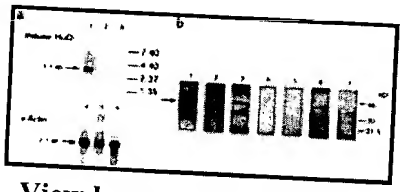

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Figure 1. (*a*) Expression of mHuD mRNA transcript in NIH/3T3 cells transfected with mHuD-expressing plasmid. Northern blot analyses of transfected NIH/3T3 cells hybridized with mouse HuD cDNA probe (*lanes 1-3*) or γ-actin cDNA probe as a control (*lanes 4-6*). *Lane 1*, NIH/3T3 cells transfected with plasmid pCMV.mHuD(+); *lane 2*, NIH/3T3 cells transfected with negative control plasmid pCMV.mHuD(−); *lane 3*, sham-treated cells; *lanes 4-6*, parallel to *lanes 1-3*, respectively. Molecular markers are present at the *right side* of *lane 3*. (*b*) Expression of mHuD protein in mouse cells. Western blot analyses of mouse brain lysates (*lanes 1* and *2*), lysates of NIH/3T3 cells (*lanes 3-5*), and lysates of Colon 26/HuD (*lane 6*) and Colon 26 (*lane 7*). The patient's serum was incubated as a first antibody in all lanes, except for *lane 2*. In *lane 2*, diluted (1:500) serum from a normal volunteer was used. *Lane 3*, NIH/3T3 cells transfected with pCMV.mHuD(+); *lane 4*, NIH/3T3 cells transfected with pCMV.mHuD(−); *lane 5*, NIH/3T3 cells without transfection; *lane 6*, HuD stable transfectant (Colon 26/HuD) cells; *lane 7*, parent Colon 26 cells. Molecular marker is to the *right* of *lane 7*. *Arrow* indicates the position of a 41-kD band from mHuD.

MTT Assay

In order to evaluate the *in vitro* growth of Colon 26/HuD mHuD transfectants, Colon 26/HuD (lot.1) or parent Colon 26 cells were harvested, washed, and seeded at a density of 5×10^4 cells/well (2.5×10^4 cells/cm^2) in 500-μl aliquots of RPMI-1640-10% FCS using 24-well culture plates (Day 0). In this assay, Geneticin was omitted from Colon 26/HuD. The culture medium was changed every 3 d for both cells. At 24 h (Day 1), on Days 2, 4, or 7 after seeding, 100 μl of sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml) was added and incubated for 3.5 h at 37°C (22, 23). After rinsing with PBS, the resulting blue formazane product was solubilized with 400 μl of dimethyl sulfoxide. The optimal density was determined in triplicate against a reagent blank (no cell) at a test wavelength of 570 nm and a reference of 630 nm. The value reflected the viable cell population in each well.

Immunization of Animals

Plasmid pCMV.mHuD(+) or (−), 75 μg, in 0.1 ml of sterile PBS was injected intramuscularly into 5-wk-old female BALB/c mice. Plasmid was injected three times at 2-wk intervals, the initial injection at Day 0, then Days 14 and 28 alternatively into either side of the quadriceps muscle.

In Vivo Tumor Models

To evaluate the feasibility of DNA vaccination against HuD antigen to suppress *in vivo* tumor growth, two protocols were used.

Protocol 1. Subcutaneous tumors were established by injection of 1×10^6 Colon 26/HuD (lot.2) cells or Colon 26 cells into the right or left flank of BALB/c mice vaccinated with either pCMV.mHuD(+) or pCMV.mHuD(−) at Day 36 after immunization. Each group consisted of four mice. This protocol was performed to evaluate the growth of Colon 26/HuD tumor, compared with the Colon 26 tumor control in the same animal. After shaving, tumor size was measured using calipers and calculated as a product of width and length. Final measurement of size was taken at the time of death.

Protocol 2. To examine the growth of Colon 26/HuD tumor without the influence of Colon 26 cells, Colon 26/ HuD cells alone were injected into the mice vaccinated with pCMV.mHuD(+) or pCMV.mHuD(−) or into sham-treated mice. Sham-treated control mice received only PBS three times at 2-wk intervals. Each group consisted of five mice. At Day 36 after vaccination, 1×10^6 of Colon 26/HuD cells (lot.3) were injected into both flanks of all mice. Tumor size was measured until 19 d after inoculation of tumor cells. Tumor size was calculated as a product of the mean width and length from both sides.

Histologic examination of brain sections from DNA-vaccinated mice was carried out. The brain was excised at Day 36 after vaccination (without inoculation of tumor cells) or 19 d after inoculation of tumor cells. Tissue sections were stained with hematoxylin and eosin or Klüver- Barrera stain to evaluate pathologic findings of paraneoplastic neuronal syndromes.

Assay of Thymidine Uptake in Spleen Cells

The spleen was excised 19 d after inoculation of tumor cells from DNA-vaccinated or sham-treated animals. Each group consisted of four mice. Single-cell suspensions of spleen cells were depleted of erythrocytes by centrifugation onto Ficoll-Conray solution M-SMF (JIMRO, Takasaki, Japan). Spleen cells were rinsed and resuspended in RPMI-1640 medium, supplemented with 10% FCS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 mM *N*-2-hydroxyethylpiperazine-*N*'-ethane sulfonic acid, and 50 μM 2-mercaptoethanol (24). Irradiated (8,000 rad) Colon 26/HuD cells were used as target cells. A mixture of 1×10^5 spleen cells and 1×10^4 irradiated target cells were cultured with 5 U/ml of human recombinant interleukin-2 in round-bottomed 96-well plates for 24 h. Tritiated thymidine (20 Ci/mmol; NEM Life Science Products, Inc., Boston, MA) was added at 1 μCi/well and incubated for a further 24 h. The intact cells were harvested using a Micro 96 Harvester 11057 (Skatron Instruments, Lier, Norway), and radioactivity from thymidine uptake for DNA synthesis was measured by a liquid scintillation counter 1450 MicroBeta (Wallac, Turku, Finland) in triplicate. Radioactivity was compared between the groups of animals vaccinated with pCMV.mHuD(+) and (−) after subtraction of the value of sham-treated mice.

Antimouse HuD Antibody Production

The cell lysate of Colon 26/HuD cells was applied to 7% SDS-PAGE and transferred to a PVDF membrane (Immobilon-PSQ). The membrane strip was blotted for 2 h at 24°C with 1:500 diluted serum obtained from DNA-vaccinated animals at Day 36 after vaccination. The patient's serum was used as a positive control, as described previously. As a second antibody, 1:5,000 diluted horseradish peroxidase-labeled goat antimouse Ig (IgA + IgG + IgM [H+L]) (Southern Biotechnology Associates, Inc., Birmingham, AL) was used for the sera of DNA-vaccinated animals, or 1:5,000 diluted peroxidase-conjugated goat antihuman IgA + IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, Inc.) for the patient's serum. The blots were incubated for 1 h at 37°C, and developed with the ECL Western blotting system (Amersham).

Data Analysis

Data are presented as means ± standard deviation. Comparison of tumor size in each group was made by one-way analysis of variance. A probability of < 0.05 was considered significant.

Results

In Vitro Expression of Mouse HuD Antigen

Northern blot analysis revealed 3.7-kb messenger RNA (mRNA) transcripts of mouse HuD antigen in NIH/3T3 cells transfected with plasmid pCMV.mHuD(+) but not in NIH/3T3 cells transfected with plasmid pCMV.mHuD(−) or NIH/3T3 without transfection (Figure 1a). NIH/3T3 cells transfected with pCMV.mHuD(−) showed no band in the Northern blot hybridized with a [^{32}P]-labeled double-stranded HuD cDNA. This would represent the instability of RNA transcripts after

transient transfection in these cells. It has been reported that the serum of the patient with PEM/PSN reacted with Hu antigens between 35 and 41 kD (25). Western blot analysis demonstrated that our patient's serum reacted with a 41-kD band in the cell lysate of mouse brain (Figure 1b, *lane 1*). Further, lower and higher molecular-weight bands were also detected. However, these bands may be nonspecific because they were also detected in serum obtained from a normal volunteer (Figure 1b, *lane 2*). Lysate of transient transfectant (NIH/3T3) or stable transfectant (Colon 26/HuD) with pCMV.mHuD(+) reacted with the same sized band when incubated with serum from the patient (Figure 1b, *lanes 3* and *6*). In contrast, no 41-kD band was detected in the lysate of NIH/3T3 cells transfected with pCMV.mHuD(−), sham treatment, or parent Colon 26 cells (Figure 1b, *lanes 4*, *5*, and *7*). Thus, HuD-expressing plasmid can induce transient expression of HuD mRNA transcripts and protein in NIH/3T3. Moreover, stably transfected Colon 26 cells could produce mouse HuD antigen.

Suppression of Tumor Growth by DNA Vaccination

Mice were divided into two groups that received either plasmid pCMV.mHuD(+) or (−). No death was observed during the vaccination. Body weight gain was comparable in each group. Subcutaneous inoculation of either Colon 26/HuD or Colon 26 cells in each side was performed at Day 36 after vaccination. In Protocol 1, subcutaneous tumor was first recognized 7 d after inoculation of cells. The growth of Colon 26/HuD tumor in pCMV.mHuD(+)- treated groups was slightly suppressed compared with that of the pCMV.mHuD(−)-treated group 11 d after the inoculation of tumor cells (Figure 2). At Day 19, the size of Colon 26/HuD tumor in pCMV.mHuD(+)-treated animals (86.6 ± 29.9 mm^2) was significantly less than that in pCMV.mHuD(−)-treated animals (195.3 ± 48.1 mm^2, $P < 0.05$). On the other hand, tumor size of Colon 26 cells in pCMV.mHuD(+)-vaccinated animals (318.2 ± 78.0 mm^2) was similar to that of pCMV.mHuD(−)-vaccinated mice (320.1 ± 87.9 mm^2, $P > 0.9$). *In vivo* growth of Colon 26/ HuD tumor was less than that of Colon 26 tumor even in the mice who received negative control plasmid (Figure 2). This might reflect the slower growth of Colon 26/HuD cells compared with that of Colon 26 cells, because MTT assay revealed that *in vitro* growth of Colon 26/HuD was slightly less than that of parent Colon 26 cells (Figure 3).

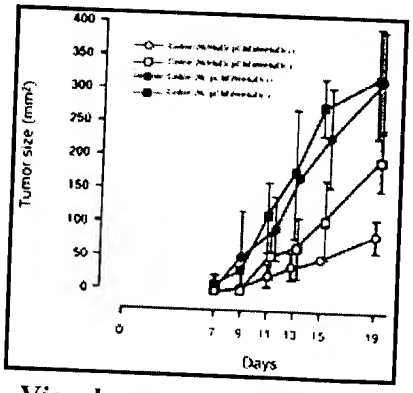

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Figure 2. Suppression of the growth of HuD-expressing cell tumors (Colon 26/HuD) in mice vaccinated with HuD-expressing plasmid pCMV.mHuD(+). Tumors were established by the injection of 1×10^6 Colon 26/HuD or Colon 26 cells subcutaneously into the right or left flank of previously vaccinated BALB/c mice (Protocol 1 in MATERIALS AND METHODS). Size of Colon 26/ HuD and Colon 26 tumors were calculated at each time point.

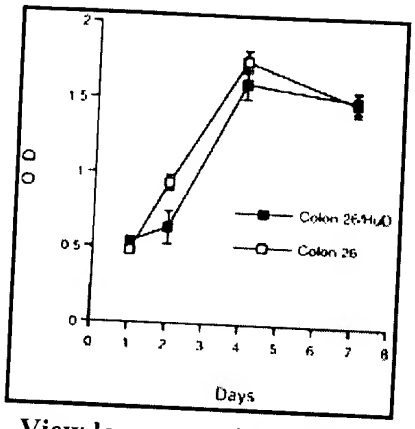

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Figure 3. *In vitro* growth of mouse HuD-expressing cells (Colon 26/HuD) and Colon 26 cells analyzed by MTT assay. Colon 26/ HuD or Colon 26 cells were seeded at a density of 5×10^4 cells/ well in 24-well culture plates (Day 0). Optical density (OD) at 570 nm subtracted from the value at 630 nm reflects the number of viable cells at each time point. Growth of Colon 26/HuD cells is slower than that of Colon 26 cells.

In Protocol 2, injection of a different lot of Colon 26/HuD cells was performed in DNA-vaccinated animals. At Day 36 after vaccination, 1×10^6 Colon 26/HuD cells were injected into both flanks of all animals. Measurement of tumor size was continued until 19 d after inoculation of tumor cells (Figure 4). Mean tumor size at Day 19 in pCMV.mHuD(+)- vaccinated mice (107.7 ± 12.8 mm^2) was significantly smaller than that in pCMV.mHuD(−)-vaccinated mice (156.6 ± 22.8 mm^2, $P < 0.05$) or in sham-treated animals (145.6 ± 10.2 mm^2, $P < 0.05$). Tumor size in the animals vaccinated with pCMV.mHuD(−) was similar to that of sham-treated animals ($P > 0.3$). In Protocol 2, tumor suppression was also observed by using a different lot of Colon 26/HuD cells without Colon 26 control cells. Thus, consistent antitumor activity of DNA vaccination was shown in two protocols against different lots (subpopulations) of tumor cells.

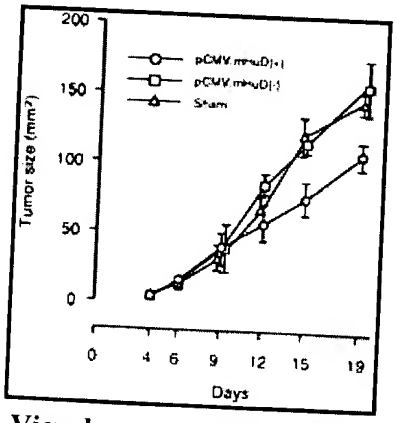

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Figure 4. Suppression of the growth of HuD-expressing cell tumors (Colon 26/HuD) in mice vaccinated with mouse HuD-expressing plasmid. Colon 26/HuD cells, 1×10^6, were injected into both flanks of animals who received DNA vaccine (Protocol 2). Tumor size was measured on 19 consecutive days after inoculation and is represented as a mean value of both sides.

Histologic examination of brain tissue obtained from the animals vaccinated by pCMV.mHuD(+) with or without inoculation of Colon 26/HuD tumor cells revealed no obvious inflammation in the cerebrum

and cerebellum (data not shown).

Thymidine Uptake of Spleen Cells

Spleen cells were obtained from mice vaccinated with pCMV.mHuD(+) or (−) or sham-treated mice, and *de novo* DNA synthesis was evaluated by incubation with Colon 26/HuD cells in the presence of tritiated thymidine. The uptake of radioactive thymidine by spleen cells obtained from the animals vaccinated with pCMV.mHuD(+) (7,212 ± 502 cpm) was significantly increased compared with that obtained from animals vaccinated with pCMV.mHuD(−) (574 ± 2,649 cpm, $P < 0.05$).

Antimouse HuD Antibody Production

Sera were collected from the animals that received either plasmid pCMV.mHuD(+) or (−) or from sham-treated mice to detect the production of anti-HuD antibody. Tumor cells were not inoculated into these mice. Each vaccinated group consisted of four animals, and individual sera were subjected to Western blot analysis. In the group of animals vaccinated with pCMV.mHuD(+), a 41-kD band was detected in all examined sera, although no similar band was seen in animals that received pCMV.mHuD(−) or in sham-treated animals (Figure 5). These results indicate that HuD-expressing DNA vaccine elicited anti-HuD antibody production in these mice.

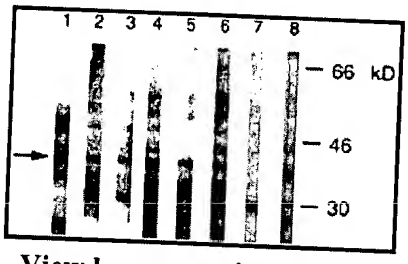

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Figure 5. Presence of anti-HuD antibody in individual sera obtained from mice immunized with HuD-expressing plasmid pCMV.mHuD(+). Lysate of HuD stable transfectants (Colon 26/ HuD) was electrophoresed in 7% SDS-PAGE and transferred to a PVDF membrane. Western blot analyses were performed with diluted (1:500) serum obtained from pCMV.mHuD(+)-vaccinated mice as a first antibody. *Lane 1*, incubation with serum of the patient with PEM as a positive control. *Arrow* indicates a 41-kD band corresponding to mHuD; *lanes 2-6*, incubation with individual sera obtained from pCMV.mHuD(+)-vaccinated mice. *Lane 7*, incubation with serum obtained from a pCMV.mHuD(−)- vaccinated mouse; *lane 8*, incubation with serum obtained from a sham-treated mouse.

Discussion

▲ Top
▲ Abstract
▲ Introduction
▲ Materials and Methods
▲ Results
• Discussion
▼ References

Although the present study shows that vaccination with HuD-expressing plasmid suppressed HuD-expressing tumor growth, the immune mechanism for the tumor suppression remains obscure. Cytotoxic T-cell killing of tumor plays a role in suppressing the tumor growth. However, antibody-dependent cell-mediated cytotoxicity is also an attractive explanation for tumor suppression from the clinical observation. The humoral immunity is related to antitumor immunity, from the observation that the presence of anti-Hu antibodies at the time of SCLC diagnosis is a strong and independent predictor of complete response to treatment and prolonged survival

(26).

Since Liu and colleagues published the sequence of human HuD antigen (in 1995), alternatively spliced isoforms of HuD have been reported among various species (27). Liu and colleagues reported two alternative spliced forms of human HuD, namely HuD*pro* and HuD*mex*. HuD*pro* contained a 14-amino-acid sequence (LMSGPVPPSACSPR) next to the 3' end of the M region of HuD (27). HuD*mex* had 13 amino acids (LDNLLNMAYGVKR) deleted from HuD (27). It has been reported that the expression of HuD*mex* is increased in human SCLCs (5). In addition, it has also been reported that an alternatively spliced form of rat HuD has an identical 13-amino-acid sequence deleted, as seen in HuD*mex* (18). We obtained a short form of the mouse HuD sequence that had 42 nucleotides deleted, identical to 13 amino acids deleted in human HuD*mex*, in the result of amplification of cDNA that originated from the RNA of mouse brain. Northern blot analysis of our mouse HuD antigen gene revealed a 3.7-kb transcript, as seen in the rat HuD gene (18). Western blot analysis of transient and stable transfectants of the HuD gene revealed the presence of an approximately 41-kD band, almost the same size as seen in recombinant human HuD (3). In this study, the plasmid based on mouse, but not human, HuD sequences was used to immunize mice because it would avoid the immune response directed to human transgene-encoded proteins in mice. In fact, it has been reported that human erythropoietin, which is 79% identical at the amino-acid level, induced antibody production in mice (28).

Immunization with purified recombinant HuD protein based on the sequence of human HuD antigen can generate a high titer of anti-HuD antibody but not cause any pathologic findings in the brain (29, 30). Of particular interest in this study, then, was whether DNA vaccination with the mouse HuD gene, with or without challenge of HuD-expressing tumor cells, caused neurologic disease in mice. It is known that DNA vaccination elicits cellular and humoral immunity (15, 16). In fact, DNA vaccination with HuD-expressing plasmid induced *de novo* DNA synthesis and anti-HuD antibody production in this study. However, evidence of encephalomyelitis was not detected in the brains of mice vaccinated with HuD-expressing plasmid before or after tumor challenge (data not shown). Although there were questions about the role of anti-Hu antibodies in the pathogenesis of PEM/PSN, the strength of the immune response in the host may influence the onset of paraneoplastic neuronal syndromes, on the basis of the observation that patients with PEM/PSN had a higher titer of anti-Hu antibodies than did patients without neurologic disorders (2). It is suggested that the distribution of HuD antigen within neurons and SCLC is not restricted to the nucleus but is also detected on the cell surface (25). The surface-expressed HuD antigen would be recognized by the host immune system and be related to the onset of a paraneoplastic syndrome and the suppression of tumor spread in patients with SCLC. Because cell-surface expression of HuD antigen depends on nuclear expression of HuD antigen, it would be important to increase total expression of HuD antigen in the cells to activate the immune system. To potentiate antitumor immunity, we have to improve the efficacy of HuD expression in tumor cells or enhance the host immune response against HuD. This improvement will also be important in the analysis of the mechanisms of PEM/PSN. Expression of HuD is high in SCLC cells (2, 3, 11), and anti-Hu immunity was a favorable prognostic indicator for patients with SCLC (26). In addition, the immunogenecity of HuD antigen is expected to be identical between humans and BALB/c mice (30). Together with these observations, our murine experiments support the hypothesis that HuD antigen is a possible candidate molecule for antitumor vaccination against SCLC. However, it is also possible that

the neurologic paraneoplastic syndromes associated with anti-HuD immunity may abrogate this therapeutic approach.

In summary, DNA vaccination with mouse HuD antigen suppressed the growth of HuD-expressing tumors in a murine SCLC model.

Footnotes

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Abbreviations: complementary DNA, cDNA; cytomegalovirus, CMV; fetal calf serum, FCS; immunoglobulin, Ig; mouse HuD antigen, mHuD; messenger RNA, mRNA; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; phosphate-buffered saline, PBS; paraneoplastic encephalomyelitis, PEM; paraneoplastic sensory neuronopathy, PSN; polyvinylidene difluoride, PVDF; small-cell lung cancer, SCLC; sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE.

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References

▲ Top
▲ Abstract
▲ Introduction
▲ Materials and Methods
▲ Results
▲ Discussion
▪ References

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Induction of Antitumor Immunity with Dendritic Cells Transduced with Adenovirus Vector-Encoding Endogenous Tumor-Associated Antigens

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Abstract

- Top
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion
- References

Dendritic cells (DCs) are professional Ag-presenting cells that are being considered as potential immunotherapeutic agents to promote host immune responses against tumor Ags. In this study, recombinant adenovirus (Ad) vectors encoding melanoma-associated Ags were used to transduce murine DCs, which were then tested for their ability to activate CTL and induce protective immunity against B16 melanoma tumor cells. Immunization of C57BL/6 mice with DCs transduced with Ad vector encoding the hugp100 melanoma Ag (Ad2/hugp100) elicited the development of gp100-specific CTLs capable of lysing syngeneic fibroblasts transduced with Ad2/hugp100, as well as B16 cells expressing endogenous murine gp100. The induction of gp100-specific CTLs was associated with long term protection against lethal s.c. challenge with B16 cells. It was also possible to induce effective immunity against a murine melanoma self Ag, tyrosinase-related protein-2, using DCs transduced with Ad vector encoding the Ag. The level of antitumor protection achieved was dependent on the dose of DCs and required $CD4^+$ T cell activity. Importantly, immunization with Ad vector-transduced DCs was not impaired in mice that had been preimmunized against Ad to mimic the immune status of the general human population. Finally, DC-based immunization also afforded partial protection against established B16 tumor cells, and the inhibition of tumor growth was improved by simultaneous immunization against two melanoma-associated Ags as opposed to either one alone. Taken

together, these results support the concept of cancer immunotherapy using DCs transduced with Ad vectors encoding tumor-associated Ags.

Introduction

The identification of tumor-associated Ags (TAAs)[2] recognized by CTL, as well as the cloning of genes encoding TAAs, has improved the prospect for cancer immunotherapy (Refs. 1, 2, 3, 4 and reviewed in Ref. 5). On the basis of this knowledge, several investigators have focused on the delivery of TAA-derived proteins/peptides or TAA genes to professional APCs, in particular dendritic cells (DCs), to elicit immune responses capable of eradicating tumor cells.

DCs are potent Ag presenters that express high levels of costimulatory molecules and are capable of activating both $CD4^+$ and $CD8^+$ naive T lymphocytes (6, 7, 8). Results obtained in several animal models have shown that DCs pulsed with defined tumor-associated peptides or with peptides eluted from the surface of tumor cells are capable of inducing an Ag-specific CTL response resulting in protection from tumor challenge and, in some instances, regression of established tumors (9, 10, 11, 12). The same type of approach has also been tested in human clinical trials with encouraging results. For example, Hsu et al. have reported that four B cell lymphoma patients infused with autologous DCs pulsed with tumor-specific Id protein all developed an Id-specific proliferative response, accompanied by complete tumor regression in two patients and partial regression in a third (13). More recently, Nestle et al. reported that melanoma patients treated with autologous DCs pulsed with tumor lysate or a mixture of CTL peptide epitopes developed cell-mediated immunity with objective clinical responses in 5 of 16 patients evaluated (14).

We have favored a gene-based rather than a peptide/protein-based approach to DC immunization, because transduction of DCs with a TAA-encoding transgene offers several potential advantages over peptide-pulsing. First, expression of an entire TAA gene circumvents the need for the identification of specific CTL epitopes within the protein since it allows for processing and presentation of all natural CTL and, possibly, helper epitopes in the context of the host's MHC type. In addition, TAA expression within DCs provides the cell with a renewable supply of Ag for presentation, as opposed to a single pulse of peptide(s), which eventually decays from the cell surface. As a result, the Ag-presenting activity of genetically modified DCs shows greater persistence (G. Linetie, S. Shankara, R. Doll, L. Eaton, B. Roberts, and C. Nicolette, manuscript in preparation).

The introduction of genetic material into DCs can be achieved with varying levels of efficacy, using techniques such as electroporation, lipid-mediated transfection, calcium phosphate precipitation, and virally mediated gene transfer (15, 16, 17). Adenoviral vectors were selected in this study since we and others have found adenovirus (Ad) to be a highly efficient and reproducible method of gene transfer into DCs (15, 17, 18, 19, 20).

It has been reported that immunization of mice with DCs transduced with Ad vector encoding a model Ag (e.g., OVA and ß-galactosidase (ß-gal)) gives rise to a specific CTL response and provides protective

and/or therapeutic immunity against tumor cells stably expressing the same Ag (17, 18, 19, 20). In this study, we investigated the ability of Ad vector-transduced DCs to induce protective immunity against endogenous tumor Ags, as opposed to foreign model Ags introduced exogenously.

The B16 melanoma tumor model was used as a test system to evaluate and characterize the immunizing activity of DCs transduced with Ad vectors encoding melanoma-associated Ags (MAAs). The B16 tumor cell line expresses the murine homologues of human MAAs such as gp100, tyrosinase-related protein (TRP)-1, TRP-2, and melanoma Ag recognized by T cells 1 (MART-1) (21, 22, 23). Accordingly, DCs derived from murine bone marrow were transduced with Ad vector encoding the human and/or murine version of two known MAAs, gp100 and TRP-2, and were tested for their ability to induce a CTL response and provide immunity against B16 melanoma tumor cells. The impact of various factors, such as the dose of DCs, nature of the MAA, CD4 activity, and preexisting immunity to Ad, on the development of antitumor immunity was investigated.

Materials and Methods

▲ Top
▲ Abstract
▲ Introduction
▪ Materials and Methods
▼ Results
▼ Discussion
▼ References

Animals and cell lines

Female wt C57BL/6 mice and C57BL/6 CD4 knockout (KO) mice were purchased from Taconic (Germantown, NY) and were used at 8–12 wk of age. Syngeneic SV40-transformed fibroblasts (SVB6KHA) have been described elsewhere (24) and were a gift from Dr. Linda Gooding (Emory University, Atlanta, GA). The YAC-1 NK cell target derived from the A/Sn mouse strain and the C57BL/6-derived EL4 lymphoma cell line were both purchased from the American Type Culture Collection (ATCC, Manassas, VA). The B16.F10 melanoma cell line syngeneic to C57BL/6 mice was obtained from the National Cancer Institute (Bethesda, MD). For injection, B16.F10 cells ($1.5–2 \times 10^4$ cells) were resuspended in PBS and delivered to the abdomen s.c. in a 100-μl volume. Tumor size was measured with electronic digital calipers three times per week, starting around day 10. Tumors ≥3 mm^2 in size were scored as positive.

Adenoviral vectors

All recombinant Ad vectors used were derived from Ad serotype 2, from which the E1 region was deleted and replaced with an expression cassette containing a CMV promoter driving expression of the transgene. The vectors encoding ß-galactosidase (Ad2/ß-gal-4) and hugp100 (Ad2/hugp100v1) contained intact E3 and E4 regions (25, 26). A second version of Ad2/hugp100 (Ad2/hugp100v2), as well as the vectors encoding enhanced green fluorescent protein (Ad2/EGFP) and murine gp100 (Ad2/mgp100) or vector lacking a transgene (Ad2/EV), possessed an intact E3 region with an E4 region modified by removal of all open reading frames and replacement with the E4 open reading frame 6 and protein IX moved from its original location (27). Finally, the Ad vector encoding murine TRP-2 (Ad2/mTRP-2) contained an intact E4 region but was deleted for E3. The E2 region was left intact in all vectors.

Adenoviral particles were gradient purified as previously described (27), and titers were determined by

end-point dilution on 293 cells using FITC-conjugated anti-hexon Ab (28).

Preparation of bone marrow-derived DCs

DCs were prepared from bone marrow essentially as described by Inaba et al. (29). Briefly, bone marrow was flushed from the tibias and femurs of C57BL/6 mice and depleted of erythrocytes with commercial lysis buffer (Sigma, St.Louis, MO). Bone marrow cells were then treated with a mixture of Abs (PharMingen, San Diego, CA) directed against CD8 (clone 53-6.7), CD4 (clone GK1.5), CD45R/B220 (clone RA3-6B2), Ly-6G/Gr-1 (clone RB6-8C5), and Ia (clone KH74), followed by rabbit complement (Accurate Chemical and Scientific, Westbury, NY) to deplete lymphocytes, granulocytes, and Ia^+ cells. The remaining cells were cultured for 6 days in six-well plates in RPMI 1640 medium (Life Technologies, Grand Island, NY), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10% FCS, and 100 ng/ml recombinant mouse GM-CSF (Genzyme, Cambridge, MA). Loosely adherent DCs were then collected, replated in 100-mm dishes, and cultured in the same medium for another 24 h after removal of contaminating nonadherent cells. This final DC population was then collected for FACS analysis and transduction with Ad vector.

For analysis of surface markers, DCs were first incubated with unlabeled Abs (PharMingen) specific for the MHC class I (clone AF6-88.5) and class II (clone AF6-120.1) molecules, the costimulatory molecules B7.1 (CD80; clone IG10) and B7.2 (CD86; clone GL-1), the adhesion molecule ICAM-1 (CD54; clone 3E2), the integrin CD11c (clone 3E2), and the myeloid surface marker CD13 (clone R3-242). The cells were then counterstained with FITC-conjugated Abs specific for the primary Ab. FACS analysis of the stained cells was performed on an EPICS Profile Analyzer from Coulter (Palo Alto, CA).

Transduction of DCs with Ad vector was conducted in six-well plates with 4×10^6 DCs/well in a 3-ml volume of RPMI 1640 medium containing 10% FCS and 100 ng/ml GM-CSF. Virus was added to the wells at a multiplicity of infection (MOI) of 500, and the DCs were collected after 18–24 h of incubation. For injection, transduced DCs were washed and resuspended in a 100-μl volume of PBS and delivered either s.c. to the abdomen or i.v. into the tail vein as specified in the text.

Mixed lymphocyte reaction and Ag-specific proliferation

To test for the ability of DCs to induce a MLR, varying numbers of bone marrow-derived C57BL/6 DCs (10^2, 10^3, 10^4) were used to stimulate 2×10^5 allogeneic BALB/c T lymphocytes isolated from spleen cells by passage through a commercial T cell purification column (R&D Systems, Minneapolis, MN). Untransduced DCs, as well as Ad2/EGFP-transduced DCs, were tested for MLR induction. In assays of Ag-specific proliferation, 2×10^5 column-purified (R&D Systems) T lymphocytes from naive C57BL/6 mice were incubated with 10^4 syngeneic DCs that were either untransduced or transduced with wt Ad2, Ad2/EGFP, Ad2/ß-gal-4 or Ad2/mTRP-2. All assay cultures were performed in triplicate in round-bottom 96-well plates in a total volume of 200 μl. The cells were cultured for 5 days at 37°C/ 5% CO_2 and pulsed with 1 μCi/well [3H]thymidine (New England Nuclear, Boston, MA) for the last 18 h of incubation. Cells were then harvested onto glass fiber filters with a 96-well plate cell harvester (Skatron Instruments, Sterling, VA), and cell-associated radioactivity was measured by scintillation counting

(LS6800 Scintillation Counter from Beckman, Fullerton, CA).

Cytotoxic T cell assay

To evaluate levels of CTL activity, spleen cells from mice in the same treatment group (three mice/group) were pooled and stimulated in vitro with syngeneic SVB6KHA fibroblasts transduced with Ad2 vector at an MOI of 100 for 24 h. Cells were cultured in 24-well plates containing 5×10^6 spleen cells and $0.8–1.5 \times 10^5$ stimulator fibroblasts per well in a 2-ml volume. Cytolytic activity was assayed after 6 days of incubation. Target cells consisted of B16 melanoma cells, EL4 lymphoma cells, YAC cells (NK cell target), and fibroblasts, untransduced or transduced with virus at an MOI of 100 for 48 h. Targets were treated with 100 U/ml recombinant mouse IFN-γ (Genzyme) for 24 h (except for YAC cells), labeled with ^{51}Cr (51-Cr; New England Nuclear) overnight (30 μCi/10^5 cells) and plated in round-bottom 96-well plates at 5×10^3 cells/well. Effector cells were added at various E:T cell ratios in triplicate. In specified instances, effector cells were incubated with a 50-fold excess of unlabeled "cold" YAC cells for 1 h before the addition of ^{51}Cr-labeled target cells to inhibit nonspecific lysis by NK cells (30). The total reaction volume was kept constant at 200 μl/well. After 5 h of incubation of effector and target cells at 37°C/5% CO_2, 25 μl of cell-free supernatant was collected from each well and counted in a MicroBeta Trilux Liquid Scintillation Counter (Wallac, Gaithersburg, MD). The amount of ^{51}Cr spontaneously released was obtained by incubating target cells in medium alone. Spontaneous release from target cells was typically below 20%. The total amount of ^{51}Cr incorporated was determined by adding 1% Triton X-100 in distilled water, and the percentage lysis was calculated as follows: % lysis = [(sample cpm - spontaneous cpm)/(total cpm - spontaneous cpm)] x 100.

Enzyme-linked immunospot assay

The presence of gp100-specific effector cells in immunized mice was also assessed in an enzyme-linked immunospot (ELISPOT) assay (31). Briefly, spleen cells from mice immunized with DCs transduced with Ad2/hugp100 or Ad2/EV were stimulated with either a known MHC class I-restricted CTL peptide epitope from hugp100 (Ref. 32 ; $hUgp100_{25–33}$-KVPRNQDWL), the homologous epitope from mgp100 ($mgp100_{25–33}$-EGSRNQDWL), or an irrelevant H-2^b-binding CTL epitope from OVA (Ref. 17 ; $OVA_{257–264}$-SIINFEKL). The peptides were synthesized by Quality Controlled Biochemicals (Hopkinton, MA) and were >90% pure by reverse phase HPLC. Peptide-stimulated spleen cells, as well as unstimulated spleen cells, were plated in the wells of 96-well nitrocellulose filter plates ($2.5–5 \times 10^4$ cells in 100 μl) coated with rat anti-mouse IFN-γ capture Ab (clone RMMG-1 from Biosource International, Camarillo, CA) and were incubated for ~48 h at 37°C/5% CO_2. The cells were then removed by washing with PBS, and the presence of IFN-γ produced by spleen cells was detected by the addition of biotinylated rat anti-mouse IFN-γ (clone XMG1.2 from PharMingen), followed by alkaline phosphatase-conjugated streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The number of stained spots corresponding to IFN-γ-producing cells was enumerated under a dissecting microscope.

Preimmunization with wt adenovirus

To generate a cohort of mice with preexisting immunity against Ad, animals were instilled intranasally

with 10^9 infectious units (i.u.) of wt Ad2, followed by a second instillation with 10^8 i.u. 14 days later. Eyebleeds were collected 1 day before the administration of DCs, and serum titers of Ad-specific Abs were assessed by ELISA. Serial 2-fold dilutions of serum were added to the wells of 96-well plates coated with heat-inactivated Ad2. Bound virus-specific Abs were detected by the addition of HRP-conjugated goat anti-mouse IgG, IgM, and IgA (Cappel, Durham, NC). The titer was defined as the reciprocal of the highest dilution of serum that produced an $OD_{490} \leq 0.1$.

Results

Characterization of bone-marrow derived DCs and transduction by Ad vectors

DCs derived from mouse bone marrow exhibited the veiled dendrite morphology typical of DCs (Fig. 1) and displayed a characteristic set of DC surface markers (33) as determined by FACS analysis (Table). The cells expressed high levels of the MHC class I and class II molecules, the costimulatory molecules B7.1 and B7.2, the ICAM-I adhesion molecule, the integrin CD11c and the CD13 myeloid surface marker. Exposure of DCs to recombinant Ad2-based vector at a MOI of 500 reproducibly resulted in a transduction efficiency of 90% or greater as determined by the percentage of DCs exhibiting fluorescence following transduction with Ad vector encoding EGFP (Ad2/EGFP). Transduction did not affect the distribution of DC surface markers significantly except for a reproducible increase in levels of MHC class I molecules (Table).

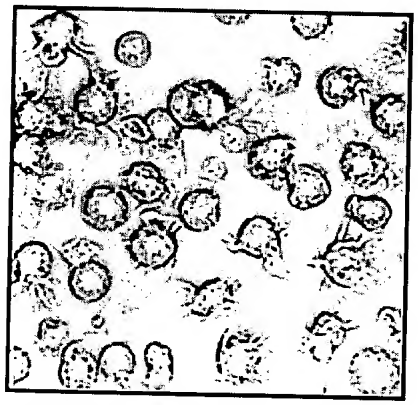

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FIGURE 1. Morphology of bone marrow-derived DCs (x200 magnification).

View this table: [in this window] [in a new window] Table I. FACS analysis of DC surface markers[1]

Bone marrow-derived DCs were functionally active in vitro, as indicated by their ability to induce proliferation of allogeneic T lymphocytes in a mixed lymphocyte reaction. This stimulatory activity was not impaired by transduction with Ad vector (Fig. 2*A*). Moreover, DCs transduced with Ad vectors encoding various transgenes were found to induce proliferation of naive syngeneic T lymphocytes, most likely due to processing and presentation of transgene products and/or Ad proteins by the DCs (Fig. 2*B*).

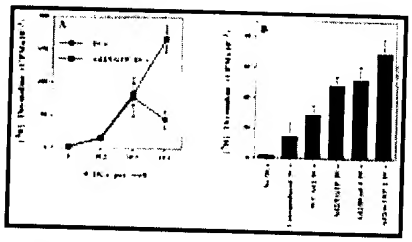

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FIGURE 2. In vitro assessment of the functional activity of bone marrow-derived DCs. *A,* In a mixed lymphocyte reaction, increasing numbers of DCs derived from C57BL/6 bone marrow (1×10^2-1×10^4 DCs) were used to stimulate 2×10^5 allogeneic BALB/c T lymphocytes. Untransduced DCs, as well as DCs transduced with Ad vector encoding green fluorescent protein (Ad2/EGFP), were tested. The levels of proliferation induced were measured by tritiated thymidine incorporation after 5 days of culture. Results shown represent the mean cpm of triplicate wells ± SEM. The background proliferation of untransduced DCs and Ad2/EGFP-transduced DCs incubated alone was highest with 1×10^4 DCs, with cpm values of 2867 ± 681 and 3392 ± 367 cpm, respectively. Results from one representative experiment are shown. A decrease in the levels of proliferation induced by the highest concentration of untransduced DCs (1×10^4) was observed in two of three experiments, and the reason for this phenomenon is unclear. *B,* To assess primary Ag-specific proliferation, naive C57BL/6 T lymphocytes (2×10^5/well) were incubated with syngeneic untransduced DCs or with DCs transduced with wt Ad2 or Ad2 vectors expressing various transgenes (10^4 DCs/well). Proliferation levels were assessed after 5 days of culture. Background proliferation of transduced DCs incubated alone was as follows: wt Ad2, 5004 ± 42; Ad2/EGFP, 1198 ± 293; Ad2/ß-gal-4, 3059 ± 1137; Ad2/mTRP-2, 920 ± 208; and untransduced DCs, 2867 ± 681 cpm. The induction of Ag-specific proliferation by transduced DCs was observed in three of three separate experiments.

Induction of tumor-specific CTL response by transduced DCs

After confirming the functionality of DCs in vitro, their ability to induce a CTL response against an MAA was evaluated in vivo. DCs were transduced with an Ad vector encoding hugp100 (Ad2/hugp100v1), a differentiation Ag that is expressed by most melanomas but is also present in normal melanocytes and pigmented cells of the retina. Ad2/hugp100v1-transduced DCs (5×10^5) were administered i.v. to C57BL/6 mice, and, 15 days later, spleens were collected for assessment of CTL activity. Separate groups of mice were also treated with vehicle as a negative control or with the Ad2/hugp100v1 vector itself for comparison. The vector was delivered under conditions previously determined to be optimal for immunization (3×10^9 i.u., intradermally (i.d.)).

After in vitro restimulation with syngeneic fibroblasts transduced with Ad2/hugp100v1, effector splenocytes were tested for cytolytic activity against ^{51}Cr-labeled target fibroblasts that were either untransduced or transduced with Ad2/hugp100v1 or wt E3-deleted Ad (Ad2Δ2.9). The CTLs were also tested against B16 tumor cells, a cell line originally derived from a spontaneously arising melanoma in C57BL/6 mice that expresses the murine equivalent of hugp100.

As expected, mice treated with vehicle failed to develop any significant CTL activity against any of the targets (Fig. 3*A*). Mice immunized with transduced DCs developed high levels of CTL activity against target fibroblasts infected with the Ad2/hugp100v1 vector. Interestingly, the bulk of the CTL response appeared to be directed against the hugp100 transgene product rather than adenoviral protein(s) since there was very little lysis of fibroblasts infected with E3-deleted wt Ad (Fig. 3*B*). A similar bias in the specificity of the CTL response toward the transgene product was also observed by Wan et al. (20) and Gong et al. (18), following immunization of mice with DCs transduced with Ad vector encoding the polyoma middle T Ag or the DF3/MUC1 tumor-associated Ag, respectively.

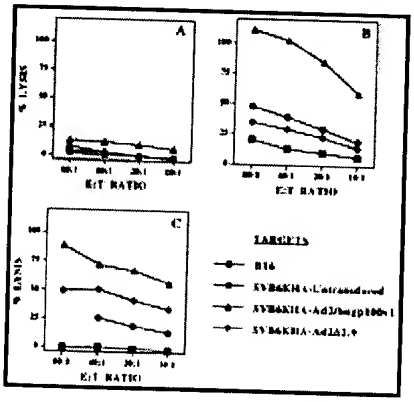

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FIGURE 3. Induction of CTL activity following immunization with Ad2/hugp100v1 vector or Ad2/hugp100v1-transduced DCs. Spleens from groups of three animals were collected 15 days after i.v. administration of vehicle (*A*), Ad2/hugp100v1-transduced DCs (*B*), or i.d. delivery of Ad2/hugp100v1 vector (*C*). Pooled spleen cells from each group were restimulated in vitro with syngeneic SVB6KHA fibroblasts transduced with Ad2/hugp100v1 and were tested for cytolytic activity after 6 days of culture. Targets consisted of B16 cells and SVB6KHA fibroblasts untransduced or transduced with Ad2/hugp100v1 or wt Ad2 deleted for E3 (SVB6KHA-Ad2Δ2.9). SD for mean percent lysis values was below 15%. Similar results were obtained in three separate studies.

Mice immunized i.d. with the Ad2/hugp100v1 vector itself, developed robust but comparatively lower levels of CTL activity against Ad2/hugp100v1-transduced fibroblasts. Furthermore, in contrast to the response obtained with transduced DCs, a significant proportion of the CTL response appeared to be specific for Ad Ag, as indicated by the greater level of lysis of fibroblasts infected with E3-deleted wt Ad

(Fig. 3C). Importantly, CTLs from mice immunized with transduced DCs and, to a lesser extent, with Ad vector were both able to lyse B16 tumor cells, suggesting that the CTLs induced by immunization with Ad2/hugp100 also recognized the endogenous mouse gp100 expressed by the tumor cells (Fig. 3, *B* and *C*). A similar cross-reactivity between the human and murine Ag was observed by Overwijk et al. following immunization of C57BL/6 mice with recombinant vaccinia virus encoding hugp100 (32).

An ELISPOT assay was used to confirm the presence of CTLs specific for gp100 since CTLs against culture medium components could potentially be present that may participate in the in vitro lysis of B16 cells. In these studies, mice were immunized with DCs transduced with Ad2/hugp100v2, or Ad2/EV as a negative control, and spleen cells were stimulated in vitro with peptide corresponding to either a known H-2^b-binding CTL epitope from hugp100 (32), the homologous sequence from mgp100 (32), or a known H-2^b-binding CTL epitope from OVA as a negative control (17). The number of class I-restricted CTLs that produced IFN-γ upon specific peptide recognition was measured after 48 h. Results shown in Fig. 4 confirm the presence of CTLs specific for hugp100 peptide in mice immunized with Ad2/hugp100v2-transduced DCs and demonstrate the extensive cross-reactivity between the human and murine epitope. As expected, spleen cells from mice that received DCs transduced with Ad2/EV show little or no reactivity against gp100 peptides, and neither group of mice shows any significant reactivity against the negative control OVA peptide.

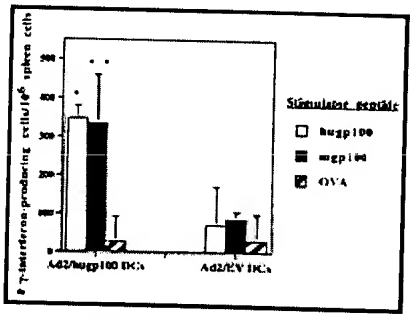

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FIGURE 4. Specificity and cross-reactivity of effector cells induced by Ad2/hugp100v2-transduced DCs. Spleen cells from mice immunized with DCs transduced with Ad2/hugp100v2 or Ad2/EV were tested in an ELISPOT assay. The number of IFN-γ-producing cells was counted after 48 h of stimulation with an MHC class I-restricted CTL peptide epitope from hugp100 (open bars), the homologous epitope from mgp100 (filled bars), or an irrelevant H-2^b-binding CTL epitope from OVA (slashed bars). Results shown are the mean number of IFN-γ-producing spleen cells ± SEM of triplicate wells after subtracting the background values obtained with spleen cells incubated alone. $*$, $p < 0.005$, and $**$, $p < 0.025$, compared with OVA-stimulated spleen cells by Student's *t* test.

Further investigation was conducted regarding the route of DC administration. As shown in Fig. 5, mice immunized with Ad2/hugp100v1-transduced DCs delivered s.c. or i.v developed similar levels of CTL activity against B16 melanoma cells with comparatively little lysis of syngeneic gp100-negative EL4 lymphoma cells. Significant lysis of the YAC NK cell target was also observed. However, NK cells could not account entirely for the lysis of B16 tumor cells since cold target inhibition with an excess of unlabeled YAC cells successfully prevented lysis of labeled YAC cells without significantly affecting specific lysis of B16 cells. These results indicate that the s.c. and i.v. routes of immunization with transduced DCs elicit equivalent levels of CTL activity against B16 tumor cells.

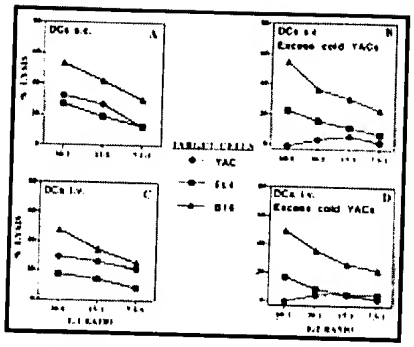

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FIGURE 5. Induction of CTL activity by transduced DCs delivered via the s.c. or i.v. route. Spleens from groups of three mice were collected 16 days after the s.c. (*A* and *B*) or i.v. (*C* and *D*) delivery of Ad2/hugp100v1-transduced DCs. Pooled spleen cells from each group were restimulated in vitro with syngeneic SVB6KHA fibroblasts transduced with Ad2/hugp100v1 and were tested for cytolytic activity after 6 days of culture. Target cells consisted of ^{51}Cr-labeled YAC cells (NK cell target), C57BL/6-derived EL4 lymphoma cells, and B16 melanoma cells incubated with effector CTLs in the presence (*B* and *D*) or absence (*A* and *C*) of excess unlabeled "cold" YAC cells. SD for mean percent lysis values was below 15%.

Antitumor protection elicited by preimmunization with Ad2/hugp100-transduced DCs

The ability of Ad2/hugp100v1-transduced DCs to induce effector CTLs capable of lysing B16 tumor cells in vitro suggested that DC immunization may also provide antitumor protection in vivo. This was first tested in a pretreatment model whereby mice were immunized with an i.v. injection 5×10^5 Ad2/hugp100v1-transduced DCs and challenged 15 days later with a lethal s.c. injection of 2×10^4 B16 tumor cells. Animals in negative control groups that were pretreated with vehicle or untransduced DCs developed rapidly growing tumors leading to death of the animals within 30 days (Fig. 6). In contrast, mice preimmunized with transduced DCs showed significant resistance to tumor growth, and only one of five animals developed a tumor with delayed kinetics. To test for the presence of immunological memory, the remaining tumor-free mice were given a second B16 challenge, 50 days after the first B16 cell injection. Three of the four mice preimmunized with transduced DCs remained tumor-free upon rechallenge, indicating that a single administration of DCs was sufficient to induce long-term antitumor immunity.

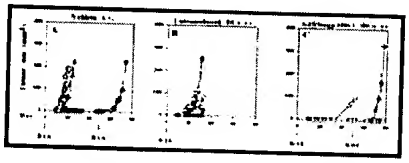

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FIGURE 6. Induction of long-term antitumor protection by Ad2/hugp100v1-transduced DCs. Groups of five C57BL/6 mice were injected i.v. with vehicle (*A*), 5×10^5 untransduced DCs (*B*), or 5×10^5 Ad2/hugp100v1-transduced DCs (*C*). The animals were challenged 15 days later with a s.c. injection of 2×10^4 B16 melanoma cells. Results shown depict tumor growth in individual animals over time. All animals that were still tumor-free 50 days after B16 challenge received a second injection of B16 cells to test for immunological memory. Results are representative of six separate studies using five to eight mice per group.

Factors involved in the effectiveness of immunization with Ad vector-transduced DCs

Nature of the Ag.

The Ad2/hugp100v1 vector expresses the hugp100 Ag, which, upon presentation by DCs, was found to elicit a protective immune response against murine B16 melanoma cells. To determine whether immunization against a homologous murine MAA would be as effective in inducing protective antitumor immunity, mice were preimmunized i.v. with DCs transduced with Ad vectors encoding the murine MAAs gp100 (Ad2/mgp100) or tyrosinase-related protein 2 (Ad2/mTRP-2). Protection from B16 tumor cell challenge administered 15 days later was compared with that obtained following immunization with Ad2/hugp100v1-transduced DCs, or untransduced DCs as a negative control. As observed previously, mice pretreated with untransduced DCs developed rapidly growing tumors whereas mice preimmunized with Ad2/hugp100v1-transduced DCs showed resistance to tumor challenge so that only two of five mice developed tumors (Fig. 7⊞*A*). In contrast, four of five mice treated with DCs transduced with Ad vector encoding the murine homologue of gp100 showed progressive tumor growth, indicating that protective immunity failed to develop in these animals. This finding is in agreement with the results of Overwijk et al., who reported that recombinant vaccinia virus encoding murine gp100 was nonimmunogenic in C57BL/6 mice (32). The failure to induce antitumor protection may have been attributed to the difficulty in breaking immunological tolerance against a self Ag as opposed to the heterologous human protein. However, mice immunized against mTRP-2 with Ad2/mTRP-2-transduced DCs did develop a protective immune response against B16 cells, and five of five mice remained tumor-free (Fig. 7⊞*A*). This result indicates that it is in fact possible to generate an effective immune response against a tumor self Ag, but that not all tumor-associated self Ags can be expected to be equally potent.

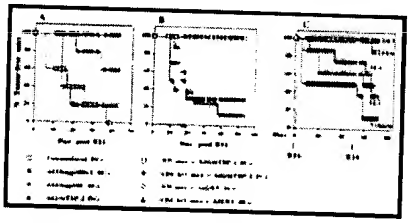

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FIGURE 7. Factors involved in the effectiveness of immunization with Ad vector-transduced DCs. *A,* Nature of the Ag: groups of five C57BL/6 mice were injected i.v. with 5×10^5 DCs that were either untransduced or transduced with Ad2/hugp100v1, Ad2/mgp100, or Ad2/mTRP-2 vector. The animals were challenged 15 days later with a s.c. injection of 2×10^4 B16 melanoma cells. *B,* Involvement of $CD4^+$ T cells: groups of eight wt or 10 CD4 KO C57BL/6 mice were immunized s.c. with 5×10^5 DCs transduced with Ad2/mTRP-2, or Ad2/EV as a negative control. The animals were challenged 14 days later with a s.c. injection of 2×10^4 B16 melanoma cells. *C,* Dose dependence: groups of eight C57BL/6 mice were immunized s.c. with increasing doses (5×10^3-5×10^6) of Ad2/mTRP-2-transduced DCs. One group received vehicle as a negative control. Animals were challenged with 2×10^4 B16 cells s.c. 15 and 64 days later. All results are shown as the percentage of tumor-free mice in each group over time.

Involvement of $CD4^+$ cells.

Overwijk et al. attributed the nonimmunogenicity of mgp100 to the low affinity of a mapped

H-2D^b-restricted CTL epitope in the murine compared with the human Ag (32). However, whereas much attention has been focused on the induction of tumor-specific CD8$^+$ CTLs, results shown in Fig. 7*B* underscore the additional importance of CD4$^+$ T cells, and consequently class II-restricted epitopes, in the development of an optimal antitumor response. In the experiment shown, wt and CD4 KO C57BL/6 mice were immunized in parallel with Ad2/mTRP-2-transduced DCs and challenged with B16 cells 14 days later. As observed above, preimmunization of wt mice with Ad2/mTRP-2-transduced DCs offered 100% protection from B16 challenge (eight of eight mice tumor-free). In contrast, only three of ten CD4 KO mice were able to inhibit tumor growth, indicating that, despite the presence of CD8$^+$ cells, the development of antitumor immunity was severely impaired in these animals (Fig. 7*B*).

Dose dependence.

The level of antitumor protection achieved by preimmunization with Ad-transduced DCs was also found to be dependent on the dose of DCs administered. As shown in Fig. 7*C*, maximal 100% protection from B16 challenge was achieved with s.c. administration of 5 x 10^5 Ad2/mTRP-2-infected DCs, with an observed decrease in levels of antitumor protection as the dose was reduced to 5 x 10^4 and 5 x 10^3 transduced DCs. Increasing the dose to 5 x 10^6 DCs did not provide any additional benefit but also failed to induce any discernible toxicity.

Immunization with Ad vector-transduced DCs in Ad-immune mice

Most individuals in the general population have been preexposed to wt Ad and are expected to possess some level of preexisting immunity against Ad. To mimic the expected clinical situation and evaluate the impact of Ad immunity on the activity of Ad-transduced DCs, mice were preimmunized intranasally with wt virus until they developed high titers of Ad-specific Abs (Fig. 8) and, as documented previously, virus-specific CTLs (34). Ad-immune and naive mice were then immunized s.c. with 5 x 10^5 Ad2/mTRP-2-transduced DCs and were challenged 15 days later with B16 tumor cells. As shown in Fig. 8, naive and Ad-immune mice developed comparable levels of tumor protection with twelve of twelve and ten of twelve tumor-free mice, respectively. Similar results were also obtained with Ad2/hugp100v1-transduced DCs delivered via the i.v. route of immunization (not shown). As expected, negative control animals, which received DCs transduced with Ad2/EV lacking a transgene, developed tumors whether the mice were naive (zero of eight tumor-free) or preimmunized against Ad (one of eight tumor-free). These results suggest that immunization with Ad vector-transduced DCs is unlikely to be impaired significantly in individuals previously exposed to Ad.

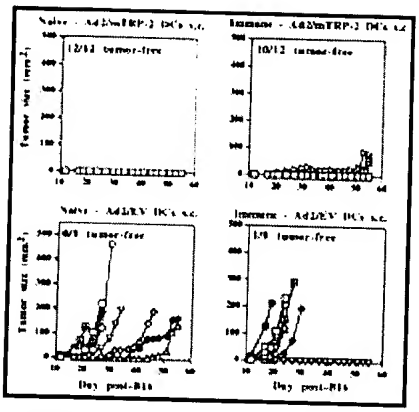

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FIGURE 8. Immunization with Ad vector-transduced DCs in animals with preexisting immunity against Ad. Groups of 12 naive or 12 Ad-immune mice were immunized s.c. with 5 x 10^5 Ad2/mTRP-2-transduced DCs. In parallel, groups of 8 naive or 8 Ad-immune mice received the same number of DCs transduced with Ad2/EV as a negative control. The ELISA titers of Ad-specific Abs present in immune serum collected the day before DC immunization ranged from 12,800 to 51,200. All mice were challenged s.c. with 2 x 10^4 B16 cells 15 days after administration of DCs. The kinetics of tumor growth are depicted for each individual animal.

Active treatment of established B16 tumor cells with Ad vector-transduced DCs

Up to 100% protection against a lethal challenge of B16 tumor cells was achieved by preimmunization with DCs transduced with Ad vector-expressing MAAs. To extend these findings to a more clinically relevant model, Ad-transduced DCs were also tested in an active treatment setting. In the study shown in Fig. 9*A*, mice received a lethal s.c injection of B16 tumor cells, which were allowed to establish themselves for 4 days before treatment with Ad-transduced DCs. As expected, negative control animals that were untreated or treated with Ad2/EV-transduced DCs were unable to control tumor growth. Mice treated with Ad2/hugp100v1-transduced DCs, which provided significant antitumor protection in a preimmunization setting, showed little or no protection from tumor growth (one of five tumor-free) in an active treatment setting. Treatment with Ad2/mTRP-2-transduced DCs, which provided 100% protection in a pretreatment setting, gave rise to partial antitumor protection with three of five mice remaining tumor-free. Therefore, an overall reduction in efficacy was seen in the more stringent active treatment model, which requires rapid mobilization of an immune response against aggressive tumor cell growth.

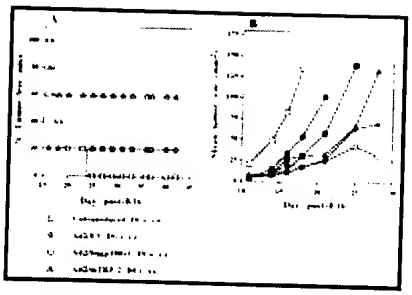

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FIGURE 9. *A,* Active treatment of established B16 tumor cells with Ad vector-transduced DCs. Twenty C57BL/6 mice were injected s.c. with 1.5×10^4 B16 cells on day 0. Four days later, the animals were divided randomly into groups of five and were treated s.c. with 5×10^5 DCs that were untransduced or transduced with Ad2/EV, Ad2/hugp100v1, or Ad2/mTRP-2 vector. Results shown represent the percentage of tumor-free mice in each group over time. Note that several animals had already developed tumors when tumor measurement was initiated on day 16. Results are representative of six separate studies using five to eight mice per group. *B,* Active treatment of established B16 tumor cells using transduced DCs presenting two vs one MAA. Forty-six C57BL/6 mice were injected s.c. with 2×10^4 B16 cells on day 0. Four days later, the animals were divided randomly into six groups, which were treated with a s.c. injection of 5×10^5 DCs transduced with either Ad2/EV (•), Ad2/hugp100v2 (■) or Ad2/mTRP-2 (▲). Two groups received a mixture of DCs transduced separately with Ad2/hugp100v2 or Ad2/mTRP-2 in the amount of 2.5×10^5 (♦) or 5×10^5 (○) of each DC population. An additional group was treated with vehicle (□) as a negative control. All groups contained eight animals, except for the vehicle control group, which was limited to six mice. Results are presented as the mean tumor size over time. Similar results were obtained in two separate studies.

As a first strategy to improve the efficacy of active treatment with Ad-transduced DCs, mice were immunized against two MAAs simultaneously in an attempt to potentiate the immune response and/or minimize escape of tumor cell variants expressing low or nonexistent levels of a given target Ag. As shown in Fig. 9*B*, combination therapy with a mixture of two DC populations transduced with Ad2/hugp100v2 or Ad2/mTRP-2 did in fact result in enhanced inhibition of tumor growth, compared with administration of either DC population alone.

Discussion

▲ Top
▲ Abstract
▲ Introduction
▲ Materials and Methods
▲ Results
• Discussion
▼ References

Results from this study support the concept of cancer immunotherapy using DCs transduced with Ad vectors encoding TAAs. Bone marrow-derived DCs transduced with Ad vector were found to retain their phenotype as determined by FACS analysis (Table 1). Transduced DCs were also functionally active in vitro as assessed in a standard mixed lymphocyte reaction and as demonstrated by their ability to induce primary Ag-specific proliferation of syngeneic T lymphocytes (Fig. 2). In vivo testing of Ad2/hugp100v1-transduced DCs demonstrated their ability to induce a specific CTL response following i.v or s.c. delivery. The appearance of CTLs specific for gp100

peptide and capable of lysing B16 tumor cells in vitro correlated with the development of protective immunity against B16 tumor challenge in vivo.

Immunization with DCs transduced with Ad vectors encoding murine MAAs (mgp100, mTRP-2), as opposed to a heterologous human Ag (hugp100), indicated that it was possible to break immunological tolerance and induce effective immunity against a melanoma self Ag using a DC-based approach. However, the two murine Ags tested differed markedly in their ability to induce antitumor immunity. Ad2/mTRP-2-transduced DCs typically provided complete protection from B16 tumor challenge whereas Ad2/mgp100-transduced DCs gave rise to little or no protective activity. It is likely that multiple factors underlie such differences in activity. For example, the number and potency of CTL (32) and/or Th epitopes within a particular protein, as well as the density of target epitopes expressed by the tumor cells, are two variables that could influence the development of an effective antitumor response. In fact, whereas much emphasis has been placed on the identification of MHC class I-restricted CTL epitopes for induction of antitumor immunity, our results indicate that epitopes recognized by $CD4^+$ T cells also play an important role. Although the murine TRP-2 Ag is known to contain an H-$2K^b$-restricted CTL epitope (22), the impaired ability of CD4 KO mice to develop protective immunity following immunization with Ad2/mTRP-2-transduced DCs (Fig. 7*B*) underscores the involvement of $CD4^+$ T cells and suggests that presentation of MHC class II-restricted epitopes, either by transduced DCs or through secondary processing of the expressed TRP-2 protein, is essential to the development of an effective antitumor response. The exact contribution of $CD4^+$ T lymphocytes in the afferent or efferent phase of the immune response remains to be determined, but the lack of class II expression by B16 cells, even after treatment with IFN-γ (not shown), suggests that $CD4^+$ T lymphocytes are more likely to function as helper cells in the induction of antitumor responses rather than as effector cells against the largely class II-negative tumor cells. In any case, the results suggest that optimal immunization protocols should incorporate the inclusion of class II-restricted epitopes in addition to class I-restricted CTL epitopes, a criterion likely fulfilled by delivery of a complete TAA gene to DCs.

The induction of antitumor immunity by Ad vector-transduced DCs was found to be dose dependent. A single dose of 5×10^3 Ad2/mTRP-2-transduced DCs was sufficient to provide a benefit compared with vehicle-treated animals, and complete protection from tumor challenge was attained with 5×10^5 transduced DCs (Fig. 7*C*). These results suggest that, in a therapeutic setting, a clinical benefit may be achievable even with relatively low doses of transduced DCs.

An important issue to consider in the context of a clinical setting is that of preexisting immunity against Ad since most individuals in the general population have been exposed to the wt virus. However, as shown in Fig. 8, immunity to Ad appears unlikely to interfere with DC-based immunization since mice preimmunized with wt Ad2 were not significantly impaired in their ability to develop antitumor immunity following injection of Ad2/mTRP-2-transduced DCs. This finding is in agreement with the results of Brossart et al., who reported that DCs transduced with an Ad vector encoding OVA were able to induce an OVA-specific CTL response in mice that had been previously immunized with two injections of Ad vector and had developed Ad-neutralizing Abs (17). Virus neutralizing Abs were not expected to interfere with DC-based immunization, but Ad-specific CTLs, which are also present in Ad-immune mice (34), have the potential to destroy Ad-transduced DCs. Even though the "half-life" of

transduced DCs administered to Ad-immune mice was not determined in this study, the observed development of antitumor protection in these animals indicates that the transduced DCs were present long enough to induce effective immunity against tumor cells. The observation that Ad vector-transduced DCs appear to induce a CTL response largely directed against the transgene product rather than Ad proteins (Refs. 18 and 20 ; Fig. 3) raises the possibility that limited presentation of Ad Ags by Ad/TAA-transduced DCs may provide some level of protection from lysis by Ad-specific CTLs.

The efficacy of Ad vector-transduced DCs was also tested in a therapeutic setting against established B16 s.c. tumor cells. The level of antitumor protection achieved was reduced, compared with that obtained in a preimmunization model. Nevertheless, a single injection of Ad2/mTRP-2-transduced DCs resulted in complete inhibition of tumor growth in an average of three of five mice (Fig. 9*A*). Several approaches are being considered to improve this outcome further. For example, simultaneous immunization against two MAAs, as opposed to a single Ag, was tested as a means to potentiate the immune response and prevent the escape of tumor variants that may express insufficient levels of a target Ag for recognition by CTLs. The results obtained support the validity of this type of approach since administration of a mixture of DCs transduced separately with Ad vector encoding hugp100 or murine TRP-2 resulted in greater levels of tumor growth inhibition, compared with either DC population alone (Fig. 9*B*). In addition, preliminary results suggest that the therapeutic efficacy of Ad vector-transduced DCs can also be enhanced by multiple administrations of DCs (40% increase in day 40 survival with three doses compared with a single dose) or by coadministration of low dose IL-2 (not shown). In agreement with the latter observation, Shimizu et al. (35) have reported recently that low dose IL-2 dramatically enhanced the antitumor response elicited by DCs pulsed with tumor lysate in a murine sarcoma model.

Overall, the data obtained in this study provide supporting evidence for the concept of melanoma immunotherapy based on the administration of DCs transduced with Ad vectors encoding MAAs. This type of approach is considered feasible in humans since protocols have been established that allow for the expansion of large numbers of DCs from peripheral blood monocytes cultured in the presence of GM-CSF and IL-4 (36, 37). The DCs obtained can be effectively transduced with Ad vector and can induce a primary CTL response against the transgene product in vitro (G. Linette, S. Shankara, R. Doll, L. Eaton, B. Roberts, and C. Nicolette; manuscript in preparation). These observations, in conjunction with the protective antitumor activity elicited by transduced DCs in the B16 melanoma model, suggest that immunization of melanoma patients with autologous DCs transduced with Ad vector expressing human MAAs may provide a therapeutic benefit.

Acknowledgments

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mgp100 and mTRP-2 cDNAs used in the construction of Ad vectors.

Footnotes

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[2] Abbreviations used in this paper: TAA, tumor-associated Ag; DC, dendritic cell; ß-gal, ß-galactosidase; EGFP, enhanced green fluorescent protein; wt, wild type; TRP, tyrosinase-related protein; mTRP, murine TRP; EV, empty vector; hugp100, human gp100 melanoma Ag; mgp100, murine gp100 melanoma Ag; KO, knockout; Ad, adenovirus; MAA, melanoma-associated Ag; MOI, multiplicity of infection; ELISPOT, enzyme-linked immunospot; i.u., infectious unit; i.d., intradermal.

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References

▲ Top
▲ Abstract
▲ Introduction
▲ Materials and Methods
▲ Results
▲ Discussion
▪ References

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Genetic Immunization of Mice Against *Listeria monocytogenes* Using Plasmid DNA Encoding Listeriolysin O[1]

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▸ Abstract

The development of protective immunity against many intracellular bacterial pathogens commonly requires sublethal infection with viable forms of the bacteria. Such infection results in the in vivo activation of specific cell-mediated immune responses, and both $CD4^+$ and $CD8^+$ T lymphocytes may function in the induction of this protective immunity. In rodent models of experimental infection with ***Listeria monocytogenes***, the expression of protective immunity can be mediated solely by the immune $CD8^+$ T cell subset. One major target Ag of ***Listeria***-immune $CD8^+$ T cells is the secreted bacterial hemolysin, listeriolysin O (LLO). In an attempt to generate a subunit vaccine in this experimental disease model, eukaryotic plasmid DNA expression vectors containing genes encoding either the wild-type or modified forms of recombinant LLO were generated and used for genetic vaccination of naive mice. Results of these studies indicate that the intramuscular immunization of mice with specifically designed plasmid DNA constructs encoding recombinant forms of LLO stimulates peptide-specific $CD8^+$ immune T cells that exhibit in vitro cytotoxic activity. More importantly, such immunization can provide protective immunity against a subsequent challenge with viable *L. **monocytogenes***, demonstrating that this experimental approach may have direct application in prevention of acute disease caused by intracellular bacterial pathogens.

▸ Introduction

The vaccination of experimental animals with plasmid DNA containing genes encoding unique proteins has proven effective in generating both humoral and cellular Ag-specific immune responses. Immunization of various species (ranging from mice to non-human primates) with unique plasmid DNA constructs encoding foreign proteins has resulted in immune responses to Ags derived from a variety of infectious agents, including influenza (1, 2, 3, 4, 5), HIV (6, 7, 8, 9, 10), rabies (11, 12), the hepatitis C and B viruses (13, 14, 15), malaria (16, 17), and mycobacteria (18, 19). Such immunization also has proven effective in inducing protective immunity in several animal models of viral disease (1, 3, 10, 11, 20, 21), as well as in the induction of specific antitumor immune responses (22, 23, 24, 25) and in the down-regulation of the expression of experimental autoimmune encephalomyelitis (26). Collectively, these observations suggest the potential application of this unique methodology to vaccine development and as a means to enhance tumor-specific cellular immunity and control autoimmune responses.

The generation of efficacious vaccines against many intracellular bacterial pathogens has proven problematic, as induction of protective immunity is evident only following recovery from sublethal infection with many microbial pathogens. Historically, experimental infection of mice with ***Listeria monocytogenes*** has evolved as the prototypic model for characterizing protective immunity to intracellular bacterial pathogens. Preliminary studies in this disease model have demonstrated that expression of protective immunity is dependent on Ag-specific T lymphocytes, which function via soluble mediators to enhance the bactericidal activity of phagocytic cells (27, 28). While $CD4^+$ immune T cells play a critical role in the development of this immune response, the expression of protective immunity can be mediated solely by $CD8^+$ immune T lymphocytes (29, 30, 31). One of the prominent Ags recognized by these $CD8^+$ T cells is the secreted *L. **monocytogenes*** hemolysin, listeriolysin O $(LLO)^3$, a protein which also functions as an essential virulence factor for this bacterial pathogen (32, 33, 34, 35, 36). Experimental studies with inbred BALB/c mice have revealed that a H2-K^d-restricted LLO-derived peptide, designated LLO_{91-99}, is a target of immune $CD8^+$ T cells that are induced following sublethal infection with *L. **monocytogenes*** (31, 37). These $CD8^+$ T cells exhibit in vitro cytotoxicity against both LLO_{91-99}-pulsed target cells and ***Listeria***-infected phagocytic cell monolayers, and also provide in vivo protection following systemic challenge with this pathogen (31, 38, 39). In addition, other *L. **monocytogenes*** Ags (including the p60 and metalloprotease proteins) also can serve as a source of H2-K^d-restricted peptide epitopes recognized by $CD8^+$ immune CTL recovered from immunized BALB/c mice (36, 38, 39, 40, 41, 42, 43).

To determine whether genetic immunization would be effective in the acute disease model of experimental murine listeriosis, we immunized BALB/c mice with eukaryotic plasmid DNA expression vectors containing either the wild type or modified forms of *hly*, the gene encoding LLO. Following a series of intramuscular immunizations, the in vivo stimulation of LLO_{91-99}-specific $CD8^+$ CTL was evaluated by in vitro cytotoxicity assays, and the development of protective immunity was assessed following lethal challenge with *L. **monocytogenes***. Results of these studies demonstrated that genetic immunization of BALB/c mice with plasmid DNA constructs encoding the wild-type LLO molecule

induced low levels of CTL in vivo, but little or no specific immunity. In contrast, immunization of mice with another plasmid DNA construct, encoding a recombinant LLO molecule containing both a substituted mammalian signal peptide sequence and a mutation resulting in reduced hemolytic activity of LLO, optimally induced LLO_{91-99}-specific CTL activity and provided protective immunity against subsequent challenge with *L. **monocytogenes***. These results demonstrate that genetic immunization with a specifically designed plasmid DNA construct can mimic the Ag-specific immune CTL response observed following sublethal infection with this pathogen. More importantly, these findings suggest that this unique immunization methodology can provide effective protection against an acute bacterial disease.

Materials and Methods

▲ Top
▲ Abstract
▲ Introduction
• Materials and Methods
▼ Results
▼ Discussion
▼ References

Bacteria

*L. **monocytogenes*** 10403 serotype 1 was originally obtained from the American Type Culture Collection (ATCC, Manassas, VA). Virulence has been maintained by repeated passage in BALB/c mice, and the LD_{50} for these inbred mice is 1×10^4 CFU (36). The generation and characterization of the LO28-W492A mutant strain of *L. **monocytogenes*** (kindly provided by Dr. Pascale Cossart, Pasteur Institute, Paris, France) has been described previously (44). Broth cultures of bacteria were established using brain heart infusion (BHI) medium (Difco, Detroit, MI).

Plasmid DNA constructs

The full-length *hly* gene was PCR amplified from *L. **monocytogenes*** DNA template using gene-specific sense (5'-CCCATGAAAAAAATAATGCTAGTTTT-3') and antisense (5'-CAATTATTCGATTGGATTATCTACTTT-3') oligonucleotide primers. The PCR product was cloned into the pCR3 plasmid vector (Invitrogen, Carlsbad, CA) and transformed into competent *Escherichia coli* TOP10F' cells according to the manufacturer's instructions. Transformants bearing plasmids containing the complete *hly* gene in both forward (pLLO6) and reverse (pLLO11) orientations, relative to the strong CMV intermediate-early promoter sequence (P_{CMV}), contained in this plasmid vector, were identified by restriction endonuclease analysis. The mutant *hly* gene construct (encoding a tryptophan to alanine change at amino acid position 492 of LLO) was PCR amplified from DNA template derived from the LO28-W492A mutant strain of *L. **monocytogenes*** and cloned into the pCR3.1 plasmid vector (Invitrogen) and analyzed as described above. *hly* gene sequences of all of the plasmid constructs were further confirmed by automated sequence analysis using a DNA Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Chimeric genes containing the signal peptide sequence derived from the gene encoding the murine tissue plasminogen activator protein (tPA) (45) fused to *hly* were constructed by PCR as follows. Truncated *hly* wild-type and W492A mutant genes (lacking the bacterial signal peptide sequence) were PCR amplified using a *Bam*HI-containing sense oligonucleotide

(5'-TCTGGATCCGATGCATCTGCATTCAATAAAG-3') and the *hly* antisense primer. The PCR products were cloned into pGEM-T plasmid vector (Promega, Madison, WI) and transformed into competent *E. coli* JM109 cells. The signal sequence from the tPA gene was PCR amplified from the pTAM2.5-a plasmid (ATCC 63042) using sense (5'-ATGAAGAGAGAGCTGCTGTGTGTACTGC-3') and *Bam*HI-containing antisense (5'-TCTGGATCCTCTTCTGAACCTCCCATGTATT-3') oligonucleotide primers. The tPA signal sequence fragment and the truncated *hly* gene fragments were separately generated using *Bam*HI/*Sph*I digestion and ligated (Rapid DNA Ligation Kit, Boehringer Mannheim, Indianapolis, IN) to form chimeric gene templates. The chimeric genes were subsequently PCR amplified using the tPA gene sense and *hly* antisense primers and cloned into PCR3.1 plasmid vector. Following transformation into TOP10F' cells, plasmids containing the chimeric genes in forward and reverse orientations, relative to the PCMV sequence, were identified by restriction endonuclease digestion and confirmed by automated DNA sequence analysis. All plasmid constructs were maintained in the *E. coli* transformants under ampicillin selection, and large-scale concentrated preparations (3.0–8.5 mg/ml) of these constructs were generated using Plasmid Giga Kits (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

Mice and immunizations

Four- to 5-wk-old female BALB/cBkl and BALB/cJ mice were purchased from B&K Universal (Freemont, CA) and The Jackson Laboratory (Bar Harbor, ME), respectively. Mice were provided unrestricted access to food and water. For active immunization with viable *L. **monocytogenes***, 6- to 8-wk-old mice received i.v. injections with 0.05–0.10 LD_{50} (~ 300–1000 CFU) in 0.2 ml of PBS. For immunization with the plasmid constructs, 6-to 8-wk-old mice received the first of a series of three i.m. immunizations (via the tibialis anterior muscles) at 3- to 4-wk intervals with ~ 100–125 μg of plasmid DNA in 50 μl of normal saline. Normal control mice received either no immunization or were immunized with either 0.2 ml of PBS (i.v.) or 50 μl of saline (i.m.).

Cell lines and synthetic peptide reagents

The J774 macrophage/monocyte cell line was maintained in antibiotic-free DMEM (Life Technologies, Grand Island, NY) supplemented with nonessential amino acids (Life Technologies) and 5% FBS (Tissue Culture Biologicals, Tulare, CA). The H2-K^d–transfected RMAS cell line (RMAS-K^d; originally obtained from Dr. Mike Bevan, University of Washington, Seattle, WA) was maintained in antibiotic-free RPMI (Life Technologies) supplemented with 10% FBS and 400 μg/ml Geneticin (Life Technologies). The peptides designated $LLO_{91–99}$ (GYKDGNEYI) and $p60_{217–225}$ (KYGVSVQDI) were synthesized at the Portland Veterans Affairs Medical Center with an Applied Biosystems Synergy apparatus using standard Fmoc chemistry. These peptides represent the major epitopes from the *L. **monocytogenes*** LLO and p60 proteins, respectively, recognized by protective $CD8^+$ T lymphocytes derived from ***Listeria***-immunized BALB/c mice (40, 41).

Activation of CTL and adoptive transfer of cells

Spleen cells obtained from normal mice, ***Listeria***-immunized mice (at 3–8 wk following sublethal infection), or plasmid DNA-immunized mice (at 22–38 days following the final i.m. injection) were

cocultured with peptide-pulsed, irradiated naive spleen cells (as stimulator cells). These stimulator cells were irradiated (3,000 rad from a ^{137}cesium source) and pulsed with either LLO_{91-99} or $p60_{217-225}$ for 1–2 h at room temperature (1×10^7 cells/ml, 5×10^{-7} M peptide in RPMI 1640 medium supplemented with 2% FBS). The stimulator cells were washed once then cocultured at 37°C with the donor spleen cell populations (from immunized mice) for 6 days. The cells were cocultured at 5×10^6 total cells per ml (at a donor to stimulator cell ratio of 1:1) in RPMI 1640 medium supplemented with 10% FBS and 23.8 mM sodium bicarbonate, 25 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (all Sigma, St. Louis, MO), and 30 U/ml recombinant human IL-2 (Tecin, Biological Response Modifiers Program, National Cancer Institute, Frederick, MD). Following culture, cells were washed twice with RPMI 1640 medium (without antibiotic), then used for adoptive transfer studies or in vitro cytotoxicity assays. In adoptive transfer experiments, groups of three to four mice served as recipients for each effector cell population, and each mouse was infused (via the lateral tail vein) with 3×10^7 viable cells in 0.2 ml of RPMI 1640 medium.

In vitro assays of cytotoxic activity

Target cells for the chromium release cytotoxicity assays consisted of chromium labeled, peptide-pulsed RMAS-K^d cells. Approximately 5×10^6 RMAS-K^d target cells were labeled with 250 μCi of $Na^{51}CrO_4$ (NEN Life Science Products, Boston, MA) for 60 min, washed twice, and then pulsed with a 10^{-9} M concentration of LLO_{91-99} or $p60_{217-225}$ for 60 min. The peptide pulsed targets were added in 100 μl volumes to 96-well round-bottom microtiter plates at 1×10^4 cells/well, and effector cells were added in a 100 μl volume at the indicated E:T ratios. Following a 4-h incubation at 37°C, 150 μl of supernatant from each well was collected, relative radioactivity (cpm) determined (Micromedic gamma counter, ICN Micromedic Systems, Huntsville, AL), and the percent lysis calculated as: 100 x (experimental cpm - spontaneous cpm)/(total cpm - spontaneous cpm). Data reported represent means of triplicate wells. Total release was determined following lysis of target cells with 5% Triton X-100 (Bio-Rad, Redmond, CA), and spontaneous release was less than 7% for all experiments.

Target cells for the CFU reduction cytotoxicity assays consisted of ***Listeria***-infected J774 cell monolayers. J774 cells were deposited at $1–2 \times 10^5$ cells/well in 24-well tissue-culture plates in 1.0 ml antibiotic-free DMEM with 5% FBS, cultured overnight, and then infected with *L. **monocytogenes*** (obtained from a log phase broth culture) at a multiplicity of infection of 2–5. After 60 min, the infected cell monolayers were washed once with sterile PBS, then covered with 0.5 ml of warm (37°C) DMEM supplemented with 5% FBS and 40 μg/ml gentamicin sulfate. Culture-stimulated effector cells were added (at indicated E:T ratios) in 0.5 ml of warm (37°C) DMEM with 5% FBS at 3–4 h after infection of the J774 monolayers. Assays were terminated 4–5 h later, and the number of intracellular bacteria remaining in each well was determined by hypotonic lysis of the J774 cell monolayers with 1.0 ml sterile distilled water, serial dilution of monolayer lysates from each well in PBS, and plating the dilutions on BHI agar (Difco). Following overnight culture at 37°C, the number of bacterial CFU per individual wells was determined. Data provided represent means of triplicate wells, and are calculated as follows: percent CFU reduction = [1 - (CFU in target cell monolayers incubated with effector cells)/(mean CFU in target cell monolayers incubated without effector cells)] x 100. For all experiments, the number of bacteria recovered from wells of ***Listeria***-infected J774 cells cultured in the absence of effector cell populations

ranged from 6.64 to 7.48 $\log_{10}$ CFU.

In vivo assay of immune protection

Levels of in vivo protection expressed by immunized mice were determined as previously described (30, 46). Briefly, groups of normal or immunized BALB/c mice received an i.v. injection with ~2 LD_{50} (~20,000 CFU) of *L. **monocytogenes*** in 0.2 ml of PBS, either simultaneous to infusion of culture-stimulated effector cell populations or at 3–4 wk following the final immunization with plasmid DNA. Control groups consisted of normal (nonimmunized) mice and mice previously immunized (4–12 wk earlier) with a sublethal injection of viable *L. **monocytogenes***. Two days following bacterial challenge, spleens were removed from individual mice and homogenized in PBS, and serial dilutions (in PBS) of the homogenates were plated out on BHI agar. Following overnight culture at 37°C, CFU per spleen of individual mice were calculated and the mean level of protection for each group determined. Protection was indicated by reduced numbers of CFU in spleens of immunized mice, and calculated as: log protection = ($\log_{10}$ CFU/spleen of test mice) - (mean $\log_{10}$ CFU/spleen of normal control group).

Statistics

Analyses of the mean (±SEM) determinations for the chromium release and CFU reduction cytotoxicity assays and for the immune protection assays were performed by ANOVA (Tukey test) using the Instat biostatistical computer program (GraphPad, San Diego, CA).

Results

Immunization of mice with plasmid DNA encoding LLO

Our initial attempts to induce LLO-specific immunity involved immunization of mice with a plasmid DNA construct containing the *hly* gene encoding the wild-type form of LLO. Mice were injected with this plasmid construct three times at 3–4 wk intervals. At 4 wk after the final injection, spleen cells obtained from these immunized mice were cocultured with irradiated, LLO_{91-99}-pulsed syngeneic spleen cells obtained from naive donors (as stimulator cells), then used as effector cells in chromium release or CFU reduction cytotoxicity assays. As a positive control, spleen cells obtained from other mice at 5 wk following sublethal infection with *L. **monocytogenes*** were cocultured with this same stimulator cell population.

Results of the chromium release assays demonstrated that low, but significant ($p < 0.05$) levels of LLO_{91-99}-specific cytotoxicity could be detected following culture stimulation of splenic lymphoid cells obtained from pLLO6-immunized mice or ***Listeria***-immunized mice (Fig. 1⊡*A*). In contrast, similar culture stimulation of lymphoid cells obtained from mice immunized with the parent plasmid lacking the *hly* gene (pCR3) or a plasmid DNA construct containing the *hly* gene in the reverse orientation (pLLO11) did not stimulate LLO_{91-99}-specific CTL. In addition, none of these effector cell populations

expressed any specific cytotoxic activity against target cells pulsed with the heterologous peptide, $p60_{217-225}$ (Fig. 1*B*).

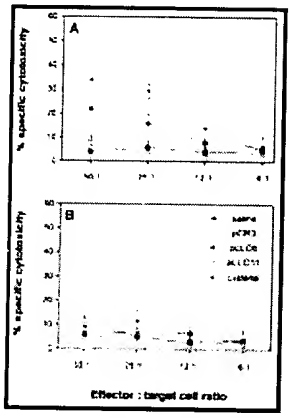

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FIGURE 1. Effector CTL derived from mice immunized with plasmid DNA containing the *hly* gene exhibit cytotoxic activity against LLO_{91-99}-pulsed target cells. Mice were immunized i.m. three times with either saline, the pCR3 parent plasmid lacking the *hly* gene, or the plasmid constructs containing *hly* in the forward (pLLO6) or reverse (pLLO11) orientation (relative to the PCMV sequence), respectively. Spleen cells were obtained from mice at 28 days following the final saline or plasmid DNA immunization or from ***Listeria***-immune mice at 35 days following sublethal infection, cocultured with LLO_{91-99}-pulsed stimulator cells, then used as effector cells (at the indicated E:T cell ratios) against chromium labeled RMAS-K^d cells pulsed with either the LLO_{91-99} (*A*) or the $p60_{217-225}$ (*B*) synthetic peptides.

These same effector cell populations derived from the plasmid DNA- or ***Listeria***-immunized mice also were assessed for the ability to specifically recognize J774 cell monolayers infected with *L. **monocytogenes***. This assay is performed by brief infection of the $H2^k$ J774 phagocytic cell line with the bacteria followed by coculture with BALB/c-derived cytotoxic effector cell populations (32, 36, 47). As observed in the chromium release cytotoxicity assays, low, but significant ($p < 0.05$), levels of cytotoxic activity could be observed following coculture of the infected J774 cell monolayers with culture-stimulated spleen cells derived from mice immunized with the pLLO6 plasmid or with viable *L. **monocytogenes***, but not with the pCR3 or pLLO11 control plasmids (Fig. 2).

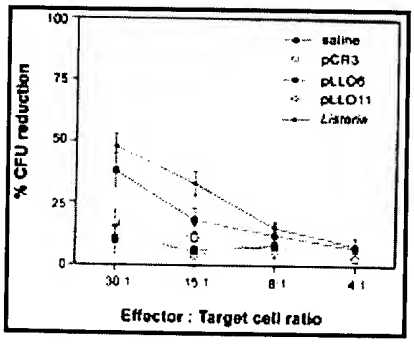

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FIGURE 2. CTL derived from mice immunized with plasmid DNA containing the *hly* gene recognize ***Listeria***-infected target cells. Culture-stimulated spleen cells obtained from plasmid DNA- or ***Listeria***-immunized mice (as described in Fig. 1) were used as effector cell populations (at indicated E:T cell ratios) against ***Listeria***-infected J774 cell monolayers. CTL recognition of ***Listeria***-infected target cells is indicated by reduced CFU recovered from individual wells of the infected cell monolayers (see *Materials and Methods* for details). The mean (±SEM) $\log_{10}$ CFU observed in wells of ***Listeria***-infected J774 cells cultured in the absence of effector cell populations was 7.01 (±0.04).

Protective immunity observed following plasmid immunization of mice

The data described above demonstrate that genetic immunization with the pLLO6 plasmid can prime LLO_{91-99}-specific cytotoxic cells in vivo. To determine whether these cells could mediate in vivo protection, effector CTL derived from either plasmid DNA- or ***Listeria***-immunized mice were evaluated for their capacity to adoptively transfer protection to naive recipients, as determined by subsequent i.v. challenge of these cell recipients with *L. **monocytogenes***.

In three of four experiments conducted, effector CTL derived from mice immunized with the plasmid DNA construct (pLLO6) containing the *hly* gene in the forward orientation could adoptively transfer low, but rarely significant, levels of protection to naive mice (Fig. 3*A*). CTL derived from ***Listeria***-immunized mice consistently provided greater, and significant ($p < 0.05$), levels of protection following cell transfer (Fig. 3*A*). Adoptive transfer of similar cell populations derived from mice immunized with the parent plasmid (pCR3) or the plasmid construct containing the *hly* gene in the reverse orientation (pLLO11) did not provide any protection. Interestingly, the levels of active immunity expressed in mice immunized with either pLLO6 or the control plasmids (pCR3 and pLLO11) were relatively undetectable (Fig. 3*B*), especially as compared with the level of immunity expressed by mice sublethally infected with *L. **monocytogenes*** at 6 wk before bacterial challenge.

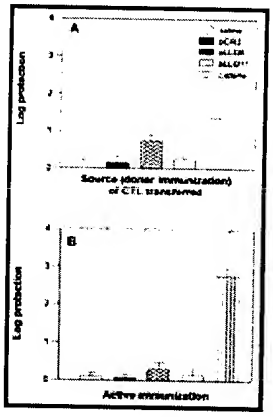

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FIGURE 3. Expression of adoptive and active immunity following immunization of mice with culture-stimulated effector cells, plasmid DNA, or viable *L. **monocytogenes***. *A*, Spleen cells derived from mice immunized with plasmid DNA or viable ***Listeria*** were stimulated in culture (as described in Fig. 1) and then infused into normal BALB/c mice. Simultaneous to cell transfer, recipient mice and normal controls received an i.v. challenge with *L. **monocytogenes***. *B*, At 34 days following the final saline or plasmid DNA immunization, or at 41 days following sublethal infection with *L. **monocytogenes***, immunized mice and normal controls received an i.v. challenge with *L. **monocytogenes***. At 2 days following *L. **monocytogenes*** challenge, the number of CFU per spleen of individual mice was determined. Data shown (log protection) represent the difference in mean $\log_{10}$ CFU recovered from spleens of normal control mice (6.69 ± 0.06) and the $\log_{10}$ CFU recovered from spleens of individual mice either infused with the effector cell populations (*A*) or immunized with saline, plasmid DNA or viable *L. **monocytogenes*** (*B*).

Generation of plasmid constructs encoding mutant and/or chimeric forms of the *hly* gene

The apparent failure to induce active immunity in experimental mice vaccinated with the plasmid DNA construct encoding LLO could be influenced by one or more factors, including 1) the inability of this vaccination methodology to induce adequate protection against a rapidly replicating, antigenically complex bacterial pathogen; 2) the suboptimal in vivo expression of the *hly* gene product, LLO, following plasmid DNA immunization; and 3) the potential toxicity of LLO for host APC following in

vivo expression of this recombinant hemolysin. Therefore, additional plasmid constructs containing mutant or modified *hly* genes were generated (see Table ⊞) for testing in this experimental disease model. One of these plasmid constructs (p492A) contained the *hly* gene cloned from the LO28-W492A mutant strain of *L. **monocytogenes***, in which a dinucleotide base pair mutation results in an amino acid change (from tryptophan to alanine) in the cholesterol binding region (amino acid position 492) of the LLO molecule (44). This mutant form of the LLO product exhibits a 100- to 1000-fold reduction in hemolytic activity relative to wild-type LLO (Ref. 44 , and data not shown), and thus in vivo expression of this recombinant LLO molecule should be less toxic to the APC. Plasmid DNA constructs encoding chimeric *hly* genes also were generated by substituting the signal sequence derived from the gene encoding the tPA for the bacterial signal sequence of the wild-type and 492A mutant *hly* genes, then subcloning these chimeric genes into the pCR3 plasmid vector. Additional plasmid constructs containing these modified genes in the reverse orientations (see Table ⊞) were developed and used as immunization controls.

View this table: Table I. Description of plasmid DNA constructs
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Immunization of mice with plasmid constructs encoding mutant and/or chimeric forms of the *hly* gene

Mice were immunized i.m. three times with the modified plasmid constructs at 3- to 4-wk intervals, and at 21–34 days following the last immunization spleen cells from donor mice were stimulated in vitro with irradiated, LLO_{91-99}-pulsed syngeneic stimulator cells. Following culture, the effector cell populations were evaluated for cytotoxic activity against peptide-pulsed target cells or against ***Listeria***-infected J774 cell monolayers. Data from these experiments demonstrated that effector CTL obtained from mice immunized with either the plasmid construct encoding the 492A mutant form of LLO (p492A) or the plasmid construct encoding the chimeric LLO molecule with the tPA signal sequence substitution (pTpaLLO6) exhibited enhanced cytotoxic activity relative to effector CTL derived from mice immunized with the plasmid construct encoding the wild-type *hly* gene (pLLO6) (Figs. 4⊞ and 5). Furthermore, effector CTL derived from mice immunized with the chimeric, mutant *hly* gene plasmid construct (pTpa492A) consistently exhibited the greatest cytotoxic activity, in some instances exceeding the cytotoxic activity observed using effector CTL derived from ***Listeria***-immunized mice (Fig. 5⊞).

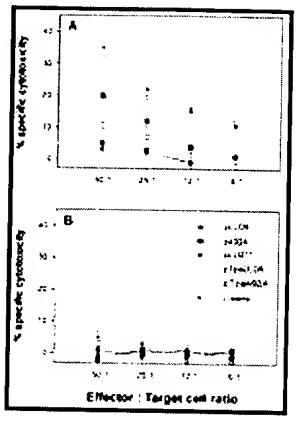

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FIGURE 4. CTL derived from mice immunized with plasmid DNA containing the mutant or chimeric forms of the *hly* gene exhibit cytotoxic activity against LLO_{91-99}-pulsed target cells. Mice were immunized with plasmid DNA constructs containing either the wild-type *hly* gene (pLLO6), the W492A mutant *hly* gene (p492A), the wild-type *hly* gene in reverse orientation with respect to the PCMV promoter sequence (pLLO11), the wild-type *hly* gene with the substituted murine tPA signal sequence (pTpaLLO6), or the W492A mutant *hly* gene with the substituted murine tPA signal sequence (pTpa492A). Spleen cells obtained from mice at 31 days following the final plasmid DNA immunization or at 38 days following sublethal infection with *L. **monocytogenes*** were stimulated in culture, then used as effector cell populations (at indicated E:T ratios) against chromium labeled RMAS-K^d cells pulsed with either LLO_{91-99} (*A*) or $p60_{217-225}$ (*B*).

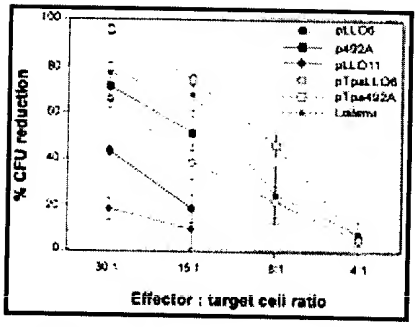

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FIGURE 5. CTL derived from mice immunized with plasmid DNA constructs containing mutant or chimeric *hly* genes recognize ***Listeria***-infected target cells. Effector CTL derived from plasmid DNA or ***Listeria***-immunized mice (as described in Fig. 4) were tested for cytotoxic activity against ***Listeria***-infected J774 cell monolayers. Cytotoxic activity (percent CFU reduction) was determined as indicated in Fig. 2. The mean (±SEM) $\log_{10}$ CFU observed in wells of ***Listeria***-infected J774 cells cultured in the absence of effector cell populations was 6.83 (±0.09).

The effector CTL populations derived from donor mice immunized with these improved plasmid constructs were further evaluated as to their ability to adoptively transfer protection to naïve syngeneic recipients. In each of four experiments, recipients of effector CTL derived from mice immunized with the plasmid DNA constructs containing the mutant or chimeric *hly* genes consistently demonstrated protection against *L. **monocytogenes*** challenge (Fig. 6*A*). Furthermore, in three of four of these experiments, effector CTL derived from mice immunized with the pTpa492A plasmid (encoding the 492A mutant LLO with the substituted tPA signal sequence) provided optimal adoptive protection to naive recipients (Fig. 6*A*). In contrast, similarly culture-stimulated spleen cells derived from mice immunized with the various control plasmid constructs (pCR3, pLLO11, $p492A_R$, pTpaLLO11, or $pTpa492A_R$) never resulted in effector cell populations exhibiting specific cytotoxicity as measured in vitro or in vivo (Figs. 4, 5, and 6*A*; other data not shown).

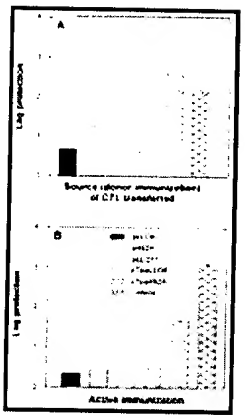

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FIGURE 6. Expression of adoptive and active immunity following immunization of mice with the mutant or chimeric *hly* plasmid DNA constructs. *A*, CTL populations derived from mice immunized with plasmid DNA or *L. **monocytogenes*** (as described in Fig. 4) were infused into normal BALB/c mice. Simultaneous to cell transfer, recipient mice and normal control mice received an i.v. challenge with *L. **monocytogenes***, and 2 days later the number of CFU per spleen of individual mice was determined. *B*, At 37 days following final immunization with plasmid DNA or at 44 days following sublethal infection with *L. **monocytogenes***, immunized mice received an i.v. challenge with *L. **monocytogenes***, and 2 days later the number of CFU per spleen of individual mice was determined. Data shown (log protection) represent the difference in mean $\log_{10}$ CFU recovered from spleens of normal control mice (6.69 ± 0.06) and the $\log_{10}$ CFU recovered from individual spleens of adoptively or actively immunized mice.

To assess levels of active immunity following vaccination with the improved plasmid DNA constructs, additional groups of mice were challenged with viable *L. **monocytogenes*** at 33–39 days following the last plasmid DNA immunization. Results of these experiments demonstrated that only mice immunized with the pTpa492A plasmid construct (encoding the 492A mutant LLO with the substituted tPA signal sequence) exhibited significant levels ($p < 0.05$) of active immunity against bacterial challenge (Fig. 6*B*). In contrast, mice immunized with the p492A plasmid (encoding the mutant LLO) or the pTpaLLO6 plasmid (encoding wild-type LLO with the substituted tPA signal sequence) exhibited either little (nonsignificant) or no protection against a lethal challenge with *L. **monocytogenes***. Mice immunized with the pLLO11, $p492A_R$, and pTpaLLO11 control plasmid constructs never exhibited any protection (Fig. 6*B*; other data not shown). Thus, these results demonstrate that immunization of naïve BALB/c mice with a plasmid DNA construct (pTpa492A) containing a mutant, chimeric form of the *hly* gene not only stimulates LLO_{91-99}-specific CTL, but also provides in vivo protection against subsequent challenge with this intracellular bacterial pathogen.

Discussion

The evaluation of DNA vaccination in this murine disease model was facilitated by previous studies characterizing the protective immune response to experimental *L. **monocytogenes*** infection. The essential role of $CD8^+$ cytotoxic T cells in this protective response, as well as the identification of a major target Ag (LLO) and a H2-K^d-restricted LLO peptide (LLO_{91-99}) recognized by these immune CTL, were critical to the evaluation of genetic immunization in this model of intracellular bacterial pathogenesis. Although our preliminary attempts to induce protective immunity with a plasmid construct (pLLO6) encoding the wild-type form of LLO were largely unsuccessful, they did suggest that LLO_{91-99}-specific $CD8^+$ CTL could be primed in vivo. These initial experimental

findings encouraged us to use other recombinant forms of the *hly* gene to facilitate this immunization event. One of these recombinant genes involved the substitution of the bacterial signal sequence of LLO with an eukaryotic signal sequence derived from the tPA gene (45). This chimeric gene was designed to facilitate enhanced in vivo expression of LLO by eukaryotic host APC populations (i.e., presumably tissue-specific macrophages and/or dendritic cells). A second *hly* gene was cloned from a mutant strain of *L. **monocytogenes*** in which a tryptophan to alanine change at amino acid position 492 (i.e., within the cholesterol-binding region of LLO) dramatically reduces the hemolytic activity of this bacterial toxin (44). This mutant gene was used to attenuate the potential toxicity of the recombinant LLO molecule expressed in vivo. A third recombinant *hly* gene incorporated both the substituted murine tPA signal sequence and the W492A mutation. Eukaryotic plasmid expression vectors containing each of these three modified *hly* gene constructs were evaluated as potential DNA vaccines in the induction of LLO-specific immunity. The data reported here demonstrate that immunization of mice with the plasmid DNA construct (pTpa492A) that encodes the less toxic LLO molecule fused to a mammalian signal sequence resulted in optimal in vivo priming of LLO_{91-99}-specific $CD8^+$ CTL and provided good protection against subsequent challenge with *L. **monocytogenes***.

The specificity of this protective immune response was indicated by the ability of CTL derived from mice immunized with the pTpa492A plasmid DNA construct to recognize *L. **monocytogenes***-infected J774 cells and to lyse RMAS-K^d cells pulsed with the LLO_{91-99}, but not the $p60_{217-225}$, peptide. In contrast, no CTL activity or in vivo protection was observed in mice immunized with plasmids containing any of the *hly* gene constructs in the reverse orientation (relative to the CMV promoter). This finding suggests that the protective response observed following immunization of experimental mice with the plasmid DNA vaccines is not a function of the innate immune response to nonmethylated CpG motifs present in the bacterial DNA (48, 49), as has been reported previously in the murine model of antilisterial immunity (50). However, as previously suggested (51, 52, 53), the presence of such immunostimulatory CpG motifs may provide an important intracellular signal that facilitates the subsequent induction of this LLO-specific immune response following immunization with the pTpa492A plasmid.

Although the level of protective immunity induced following immunization with the pTpa492A plasmid construct does not approach that observed following sublethal infection with viable *L. **monocytogenes***, it approximates the level of protection observed following immunization of mice with a recombinant strain of *Bacillus subtilis* expressing LLO (35). Presumably, the protective immune response observed in mice immunized with the pTpa492A plasmid or the recombinant *B. subtilis* is mediated solely by LLO-specific T cells. In contrast, protective immunity induced following infection with viable *L. **monocytogenes*** represents the cellular response to several bacterial Ags, including LLO as well as the p60 and metalloprotease proteins of *L. **monocytogenes***. These latter proteins also function as a source of H2-K^d-restricted peptides recognized by immune $CD8^+$ CTL in the BALB/c murine model of experimental listeriosis (31, 37, 38, 40, 41, 42, 43, 54). In addition, BALB/c mice can be immunized successfully with a *L. **monocytogenes*** mutant (designated 92F) containing a disrupted LLO_{91-99} peptide epitope (a tyrosine to phenylalanine change at amino acid 92, which inactivates this amino acid anchor residue for binding to the K^d MHC class I molecule), further suggesting that other *L. **monocytogenes***

Ags can function as targets of the protective immune response (39). Thus, the significant level of protection (i.e., ranging from 1.5 to 2.5 $\log_{10}$ reduction in CFU per spleen) observed in mice immunized with the pTpa492A plasmid DNA construct is rather remarkable, as this response is induced in the absence of bacterial infection and is directed at a single Ag expressed by a rapidly dividing, intracellular bacterial pathogen. The data provided here also demonstrate that the H2-K^d restricted LLO_{91-99} epitope is a major target of the immune $CD8^+$ cytotoxic T cells induced following immunization with the pTpa492A plasmid. However, whether such immunization also induces the MHC class Ib restricted immune $CD8^+$ T cell response previously described in this murine model of experimental listeriosis (55) remains to be determined.

Recently, Uchijima et al. (56) have reported the successful immunization of mice with plasmid DNA constructs containing an oligonucleotide sequence encoding only the LLO_{91-99} epitope. Interestingly, they could not stimulate LLO_{91-99}-specific $CD8^+$ CTL nor induce in vivo protection using the wild-type bacterial codon sequence; that is, this immunization was successful only when the LLO_{91-99} sequence information was provided by substituted codons frequently found in murine genes. Their findings contradict an earlier report (57) describing immunization of BALB/c mice with a bacterial codon-encoded LLO_{91-99} minigene expressed in a recombinant vaccinia viral vector. The data we report here further demonstrate that in vivo activation of LLO_{91-99}-specific $CD8^+$ CTL and induction of protective immunity can be effected following immunization with plasmid DNA containing the bacterial codon-encoded form of LLO, albeit the expression of this Ag is regulated by a mammalian signal sequence. Thus, our findings demonstrate that the modification of nucleotide sequences to contain mammalian-specific codons need not be a prerequisite for the application of plasmid DNA vaccination in prevention of bacterial disease. In addition, our data demonstrate the nascent development of the LLO_{91-99}-specific CTL responses following immunization with plasmids containing the full-length *hly* gene. This obviates the need to generate plasmid DNA vectors containing multiple nucleotide sequences encoding several peptides (derived from a single protein Ag) to accommodate the binding motifs of different MHC class I alleles, and thereby suggests the favorable application of this immunization methodology to outbred mammalian populations.

A number of laboratories are conducting studies directed at enhancing the efficacy of plasmid DNA immunization, either via improved plasmid DNA vectors, incorporation of multiple recombinant Ags or peptides, or co-injection of plasmid DNA constructs encoding cytokines that influence the immune response. Using one or more of these approaches, it may be possible to further augment the induction of protective immunity to *L. **monocytogenes*** following immunization with plasmid DNA constructs encoding wild-type or modified forms of LLO or other *L. **monocytogenes*** proteins. These studies should continue to provide information essential for the eventual application of this unique immunization methodology in the induction of protective immunity to other intracellular bacterial pathogens causing acute or chronic disease in mammalian populations.

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Footnotes

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[2] Address correspondence and reprint requests to Dr. R. Barry, Immunology Research P3-RD4, VA Medical Center, P.O. Box 1034, Portland, OR 97207. E-mail address:

[3] Abbreviations used in this paper: LLO, listeriolysin O; LLO_{91-99}, peptide corresponding to amino acids 91–99 of LLO; BHI, brain heart infusion; $p60_{217-225}$, peptide corresponding to amino acids 217–225 of the p60 protein of *L. **monocytogenes***; tPA, murine tissue plasminogen activator protein; PCMV, the CMV strong intermediate-early promoter sequence.

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References

▲ Top
▲ Abstract
▲ Introduction
▲ Materials and Methods
▲ Results
▲ Discussion
▪ References

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Poliovirus replicons encoding the B subunit of *Helicobacter pylori* urease elicit a Th1 associated immune response

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Abstract

The development of a vaccine for *Helicobacter pylori* is a key strategy for reducing the worldwide prevalence of *H. pylori* infection. Although immunization with recombinant B subunit of *H. pylori* urease (ureB) has yielded promising results, for the most part, these studies relied on the use of strong adjuvant, cholera toxin, precluding the use in humans. Thus, the development of new vaccine strategies for *H. pylori* is essential. Previous studies from our laboratory have described a vaccine vector based on poliovirus in which foreign genes are substituted for the poliovirus capsid genes. The genomes encoding foreign proteins (replicons) are encapsidated into authentic poliovirions by providing the capsids *in trans*. To test the utility of replicons as a vaccine vector for *H. pylori*, a replicon was constructed which encodes ureB. Expression of ureB in cells from the replicon was demonstrated by metabolic labeling followed by immunoprecipitation with anti-urease antibodies. To investigate the immunogenicity of the

replicons, mice containing the transgene for the receptor for poliovirus were immunized via the intramuscular route. Mice given three doses of replicons did not develop substantial antibodies to ureB as determined by Western blot analysis using lysates from *H. pylori*. In contrast, mice given two doses of replicon followed by a single injection of recombinant ureB developed serum antibodies to ureB which were predominately IgG2a. Splenic lymphocytes from mice immunized with replicons alone, or replicons plus recombinant ureB produced abundant interferon-γ and no detectable interleukin-4 upon stimulation with recombinant ureB. These results establish that poliovirus replicons encoding *H. pylori* ureB are immunogenic and induce primarily a T helper 1 associated immune response.

Author Keywords: Poliovirus replicon; *Helicobacter pylori*; Urease; Th1–Th2 immune responses

Index Terms: gram negative infection; immunization; bacterial vaccine

Article Outline

1. Introduction

Helicobacter pylori is a non-invasive, Gram-negative bacterium that causes chronic superficial gastritis and peptic ulcer disease and is associated with gastric carcinoma [4 and 29]. Although antibiotic treatment for *H. pylori* infection has resulted in the eradication in the infected host, the economics of treatment in developing countries coupled with the potential for emergence of resistance has necessitated the development of alternative strategies. A vaccine against *H. pylori* would be a cost effective way to prevent the late and possible life threatening disease manifestations. There have been considerable efforts toward the development of a vaccine using *H. pylori* urease, a major protein constituent of *H. pylori* which is involved in the pathogenesis of *H. pylori* infection [6, 8, 11 and 12]. A great challenge in the development of vaccine strategies for *H. pylori* is to stimulate a mucosal immune response to this

pathogen. Towards this goal, recombinant urease produced in *E. coli* protects mice from challenge with *Helicobacter* spp. when administered orally with cholera toxin [7, 9, 13, 14 and 25]. Although results of these studies serve as an important proof of concept, in humans, the oral administration of mucosal adjuvants such as cholera toxin or heat labile *E. coli* enterotoxin might result in an unacceptable level of diarrhea [10 and 15]. To circumvent this problem, recently genetically engineered mutants of heat labile *E. coli* enterotoxin [2] or cholera toxin [30 and 31] have been made to eliminate toxicity. Oral administration of recombinant *H. pylori* urease in combination with *E. coli* heat-labile enterotoxin has recently been shown to protect against *H. pylori* challenge in non-human primates [3].

Genetically engineered poliovirus offers many attractive features for development as a vaccine vector for mucosal immunization. Poliovirus is transmitted orally and enters immunoreactive mucosal sites. Moreover, the virus is available in an attenuated form that is both safe and effective as an oral vaccine [24]. To exploit the potential of poliovirus as a vaccine vector, our laboratory has developed recombinant genomes (replicons) in which a foreign gene is substituted for the genes encoding the capsid. Replicons undergo a process of RNA amplification and express foreign protein upon introduction into cells. Importantly, since replicons do not encode capsids, they cannot spread from cell to cell and therefore do not cause disease. To encapsidate these replicons, a recombinant vaccinia virus that expresses the capsid proteins of poliovirus (VV-P1) is used to provide the capsid protein *in trans* [20]. Previous studies from this laboratory have described immunogenicity of replicons encoding the C fragment of tetanus toxin [19] or HIV antigens [20]. In both studies, replicons were given to transgenic mice which contain the receptor for poliovirus and resulted in production of serum antibodies to the foreign protein.

To determine if poliovirus based vectors would be suitable as a vector for *H. pylori* proteins, we have constructed poliovirus replicons that encode the *H. pylori* urease B subunit (ureB). Expression of the ureB protein from cells infected with replicons encoding ureB was confirmed using immunoprecipitation with specific antibodies. Immunization of transgenic mice susceptible to poliovirus with ureB was used to investigate the immune response. The results of this study point to the continued development of poliovirus replicons as a vaccine vector for *H. pylori*.

2. Materials and methods

2.1. Construction of the ureB-poliovirus genome

The plasmid (pHP 902) containing the entire gene of the urease B subunit of *H. pylori* (strain UMAB 41, $CagA^+/VacA^+$) was a kind gift of Dr. H.L. Mobley, University of Maryland. Using DNA primers 5'-CTCGAG AAAAAGATTAGC AGAAAAGAATATG-3' (5' oligomer) and 5'-GTTAAC CTTTATTGGCTGGTTTAGAG-3' (3' oligomer), the urease gene was amplified by polymerase chain reaction (PCR). DNA primers were chosen to create unique *Xho*I (5') and *Hpa*I (3') restriction sites (underlines), respectively. Following amplification, the DNA product was cloned into the plasmid pCRII (Invitrogen, San Diego, CA), digested with *Xho*I and *Hpa*I and then ligated into a replicon cDNA [21] previously digested with *Xho*I and *Sna*BI enzymes; the resulting plasmid was named pT7-IC-VP4-ureB.

2.2. Encapsidation and serial passage of replicon containing the urease B subunit of *H. pylori*

The encapsidation and serial passage of encapsidated poliovirus replicons using recombinant VV-P1 has been described previously [20]. Briefly, HeLa cells grown in Dulbecco's Modified Eagle's Medium

(DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Biocell Laboratories, Rancho Dominguez, CA) were infected with VV-P1 at a multiplicity of infection of 10. After 2 h, the cells were transfected with replicon RNA or infected with encapsidated poliovirus replicons. Cultures were harvested 24 h after transfection by three successive freeze–thaws and clarified by centrifugation at 14,000×*g* for 20 min. The supernatants were stored at -70°C or used immediately for additional passages [20]. To detect poliovirus proteins or *H. pylori* ureB, the transfected and/or infected cells were metabolically labeled and the proteins were immunoprecipitated with rabbit antibodies to either poliovirus RNA polymerase (anti-$3D^{Pol}$) or *H. pylori* urease [5], followed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and fluorography as previously described [20].

2.3. Immunization of mice

Previous studies from this laboratory have shown that intramuscular immunization of mice containing the gene for poliovirus receptor (PVR1-27 transgenic mice) [23] with replicons induced serum antibodies to the foreign protein encoded by the replicon [19] (PVR1-27 mice are not susceptible to orally administered poliovirus, therefore the replicons are administered intramuscularly). A group of five twelve week-old PVR1-27 mice (Lederle–Praxis Laboratories, Rochester, NY) were immunized with 10^7 infectious units of replicons encoding ureB and boosted on days 21 and 35 with the same amount of infectious units of the designated replicon (Group C). A second group of mice were immunized with ureB replicon on day 0, boosted on day 21, but for the last boost (day 35) received 5 μg recombinant ureB protein (prepared as described in the following section) (Group D). Control groups included mice immunized once with 5 μg recombinant ureB protein on day 0 and bled 7 d later (Group B); mice immunized with 10^7 infectious units of control replicon encoding the L1 protein of human papillomavirus type 11 (HPV11) on days 0, 21 and 35 (Group A); and mice immunized three times (days 0, 21, 35) with 5 μg recombinant ureB protein (not shown). Blood was collected from the tail vein before each immunization and on day 42 (7 d after the final boost) from each mouse.

2.4. Antigens for antibody detection and measurement of antibody

2.4.1. Western Blot

H. pylori strain SPM 326 (a kind gift of Dr. Paolo Ghiara, Biocene, Siena, Italy) were cultured under microaerobic conditions at 37°C on blood agar plates, scraped into an Eppendorf tube, washed with ice cold phosphate buffered saline (PBS), counted by optical density and resuspended in PBS (approximately 1×10^{10} bacteria/ml). *H. pylori* were pelleted and boiled for 5 min in SDS–PAGE sample buffer (25 mM Tris, pH 6.8, 4% SDS, 0.01% bromphenol blue and 10% 2-mercaptoethanol). After SDS–PAGE and transfer to a nitrocellulose membrane, the blot was incubated in 3% non-fat milk/TEN (0.05 M Tris, 0.005 M EDTA, 0.15 M NaCl, pH 7.2) buffer overnight at 4°C, washed with water, air dried and then cut into 2 mm wide strips. The strips were incubated with mouse sera diluted 1:200 in 1% non-fat milk/TEN buffer for 2 h at room temperature, washed 4 times with TEN, incubated for another 1 h with peroxidase-labeled rabbit anti-mouse IgG washed again and then visualized by chemiluminescence (Enhanced Chemiluminescence Kit, Amersham, Arlington Heights, IL).

2.4.2. ELISA

To quantitate anti-ureB antibodies, we developed an ELISA using recombinant *H. pylori* ureB protein as antigen. To obtain sufficient quantities of the protein, the ureB gene was cloned into pET-16b vector (Novagen, Madison, WI) with a 10×His tag at the amino terminus. Competent *E. coli* cells, [strain

BL21(DE3)], were transformed with pET-16b-ureB and, after 3 h, the cells were induced with IPTG (1 mM) and grown for another 3 h. Urease was purified from guanidine–HCl disrupted *E. coli* using Ni–NTA resin (Qiagen, Chatsworth, CA), concentrated through a XM-50 nitrocellulose filter (Amicon, Beverly, MA) and then dialyzed against PBS (pH 6.5). ELISA plates were coated with recombinant ureB protein (5 μg/ml) overnight at room temperature, blocked with 0.25% bovine serum albumin for 2 h and then incubated with mouse serum (diluted 1:100) for 3 h. The second antibody (1:4,000) was peroxidase-labeled goat anti-mouse IgG, IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) incubated for 1 h and wells were then developed with peroxidase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Reaction was stopped with 1 M sulfuric acid and read at 450 nm.

2.5. Preparation of splenic T lymphocytes and analysis of interferon-γ (IFN-γ) and interleukin-4 (IL-4) production

Single cell suspensions were prepared from whole spleens of individual test and control mice and centrifuged at 400×*g* for 8 min at 4°C. The pellets were washed twice in 10 ml sterile RPMI (Cellgro, Mediatech Inc., Herndon, VA) containing 10% fetal calf serum (Atlanta Biologicals, Norcross, GA) with 200 U/ml penicillin and 100 μg/ml streptomycin and brought to a final volume of 10 ml on ice. The total cell count for each sample was determined by Coulter counter analysis (Coulter Electronics Ltd., Luton, England). Optimum assay conditions were determined in preliminary experiments using cells from similarly treated transgenic mice. Cells were cultured in duplicate at 4×10^5 cells/200 μl/well in 96 well plates (Costar, Cambridge, MA) in the presence of 0, 5 or 25 μg/ml recombinant ureB protein or 1 μg/ml Con A (Sigma, St. Louis, MO) for 72 h at 37°C. Culture supernatants were harvested, centrifuged at 8000×*g* for 5 min at 4°C and frozen at -70°C. Cytokine levels in the supernatants were measured by double antibody ELISA using IFN-γ and IL-4 ELISA kits (R&D Systems, Minneapolis, MN). Each supernatant was assayed in duplicate and a standard curve of known recombinant cytokine was included on every plate. Data were plotted as mean±standard error of the mean.

3. Results

3.1. Construction of the replicon encoding the ureB subunit of *H. pylori* urease

The poliovirus cDNA was reconstructed so that the foreign gene can be substituted for the genes encoding the VP2, VP3 and VP1 capsids of poliovirus (P1 region) (Fig. 1A). The foreign gene is positioned to maintain the translational reading frame between VP4 and the remaining polio proteins. To construct a replicon containing the *H. pylori* ureB gene, PCR primers were designed to amplify the DNA sequence containing the open reading frame of ureB (nucleotides 780–2453) [1]. The translational reading frame of ureB was maintained with the remaining VP4 and the P2–P3 region of the poliovirus genome (Fig. 1B) so that expression of the ureB protein from the replicon would be a VP4-ureB fusion protein (72 kDa).

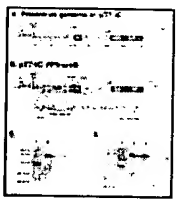
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Fig. 1. Schematic representation of a poliovirus replicon that encodes the B subunit of *H. pylori* urease. (A) Poliovirus genome. The complete cDNA of poliovirus in the plasmid designated pT7-IC [20]. (B) Replicon encoding the 62 kDa subunit of *H. pylori* urease (ureB). The ureB gene was amplified by PCR and subcloned into the vector pCRII. The oligonucleotides were chosen so that unique *Xho*I and *Hpa*I restriction endonuclease sites were added to the 5' and 3' end of the gene, respectively. The ureB gene was isolated by restriction digestion with *Xho*I and *Hpa*I and subcloned into the poliovirus cDNA, resulting in a deletion of the coding region for VP2, VP3 and VP1 in the poliovirus genome. The resulting plasmid, designated pT7-IC-VP4-ureB, contains the ureB gene positioned between the genes encoding VP4 and 2A, thereby conserving the translational reading frame. (C and D) Expression of poliovirus-specific and *H. pylori*-specific proteins in HeLa cells. HeLa cells were infected with the encapsidated replicons. The cells were metabolically labeled and immunoprecipitated as described in Section 2. For panel C, the order of the samples are as follows: lane 1 corresponds to proteins from control non-infected HeLa cells; lane 2 to proteins from infected cells with the ureB replicons; both lanes were immunoprecipitated with antibodies to poliovirus 3CD. For panel D, the order of the samples are as follows; lane 1 corresponds to proteins from control uninfected HeLa cells: lane 2 to proteins from cells infected with replicons encoding L1 capsid protein of human papilloma virus type 11 (LI HPV11): lane 3 to proteins from infected cells with the ureB replicons immunoprecipitated with rabbit anti-urease antibodies. The molecular mass markers are noted (kDa).

3.2. Expression of VP4-ureB

Transfection of VP4-ureB replicon RNA into HeLa cells resulted in the expression of a 72 kDa protein that was detected by immunoprecipitation with antibodies to the poliovirus RNA polymerase (data not shown). This is the predominant molecular form of the polymerase (designated 3CD) present in transfected (or infected) cells and the level of the enzyme correlates with the replication of the replicon [20]. The urease specific protein was also immunoprecipitated with rabbit anti-urease antibodies as a protein with molecular mass of approximately 72 kDa, consistent with the VP4-ureB fusion protein (data not shown).

The replicon was encapsidated by serial passage in the presence of VV-P1 and the encapsidated replicons were used to infect HeLa cells. Following metabolic labeling, 3CD was immunoprecipitated using anti-$3D^{Pol}$ antibodies from the lysate from replicon-infected cultures (Fig. 1C). The levels of 3CD expression were used to estimate the titer of the encapsidated replicons [20]. Since the replicons do not form plaques like wild type poliovirus, we refer to the titer in infectious units (an infectious unit correlates directly with plaque forming units for poliovirus). The cell extract was also incubated with anti-urease antibodies. We again detected a protein with a molecular mass of 72 kDa, corresponding to the VP4-ureB fusion protein and a second unknown protein of molecular mass approximately 28 kDa that could represent a proteolytic breakdown product of VP4-ureB (noted by the question mark) (Fig. 1D).

3.3. Antibody responses to immunization with the replicon encoding *H. pylori* ureB

To characterize the immune response to the ureB replicon, sera from immunized and control mice were analyzed for anti-urease antibodies by Western blot and ELISA. The Western blot analysis demonstrated that serum from mice immunized with ureB replicons only (Fig. 2C) contained no detectable antibodies to *H. pylori* urease of the dilution tested. However, when the day 35 boosted immunization was replaced with recombinant ureB protein (Fig. 2D), serum anti-urease antibodies were detected. In sera of control groups of mice, which were immunized with replicons expressing L1 capsid protein of HPV11 (Fig. 2A) or with one inoculation of recombinant ureB (Fig. 2B), anti-urease antibodies were not detected either using a Western blot or ELISA. The level of antibodies as detected by ELISA increased when the second boost (day 35) was replaced with recombinant ureB protein. More importantly, these antibodies were exclusively of the IgG2a isotype, consistent with Th1 type immune response (Fig. 3). Thus, vaccination with ureB replicons appeared to prime the mice for an enhanced Th1 immune response to

the ureB protein. Sera of animals immunized with 3 doses of ureB protein showed the most intense bands, corresponding to antibodies to the 62 kDa urease subunit (data not shown). Isotype analysis of these antibodies revealed they were predominantly of the IgG1 isotype.

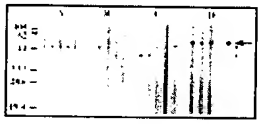
(17K)

Fig. 2. Western blot analysis of sera from (Group A) mice immunized three times with polio replicons expressing L1 capsid protein of HPV11; (Group B) mice immunized once with recombinant ureB protein; (Group C) mice immunized three times with replicons encoding ureB protein; and (Group D) mice immunized twice with replicons encoding ureB and boosted once with recombinant ureB protein. Sera are from day 42 (7 d after second boost), except for Group B mice which were bleed 7 d after the single injection of 5 μg of recombinant ureB. Each strip represents serum analyzed from an individual animal. The urease of *H. pylori* is designated with an arrow. Sera from immunized mice reacted also with undefined proteins in the *H. pylori* lysate (stars). The antigen for Western blot was lysate of *H. pylori* prepared as described in Section 2 and second antibody was peroxidase-labeled rabbit anti-mouse IgG.

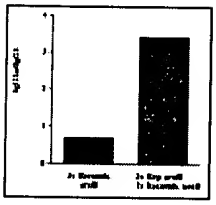
(10K)

Fig. 3. Ratio of IgG2a/IgG1 of anti-urease antibodies in the sera of mice immunized 3 times with recombinant ureB or twice with ureB replicons and once with recombinant ureB.

3.4. Cytokine responses to immunization with replicon encoding *H. pylori* ureB

The results of the isotype analysis of the immunoglobulins suggested that replicons stimulated predominantly Th1 response. To confirm this possibility, the splenic T lymphocyte production of IFN-γ and IL-4 was measured with and without stimulation with ureB protein or the mitogen concanavalin A (data not shown). Increased production of urease-stimulated IFN-γ was detected in cultures of cells from mice vaccinated with the ureB replicons and recombinant ureB (Fig. 4, column 4) compared with splenocyte cultures from control animals (Fig. 4, columns 1, 2 and 3) in which negligible levels of cytokine were detected. The highest levels and clearest dose-dependence for INF-γ production was in the splenocyte cultures from mice given two doses of replicon followed by recombinant ureB. Analysis of splenic cultures of replicons immunized animals revealed little or no IL-4 production, consistent with the notion that the immunization of mice with ureB replicons induced a strong Th1-type cytokine response by splenic T cells.

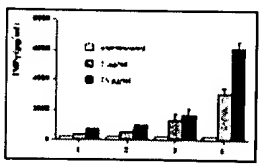
(5K)

Fig. 4. Splenic T lymphocyte production of IFN-γ. Cultures of splenocytes were assayed for IFN-γ in the absence or presence of (5 or 25 μg) recombinant ureB protein. (1) unimmunized mice; (2) mice immunized

three times with polio replicons expressing L1 capsid protein of HPV11; (3) mice immunized three times with replicons encoding ureB protein; (4) mice immunized twice with replicons encoding ureB and boosted once with recombinant ureB protein. Data are presented as mean±standard error of the mean.

4. Discussion

In this study, we have constructed and characterized a non infectious poliovirus replicon that encodes the 62 kDa subunit of *H. pylori* urease. Infection of HeLa cells with ureB replicon resulted in expression of a 72 kDa VP4-ureB fusion protein which could be immunoprecipitated with antibodies to urease. Mice given three doses of replicon via the intramuscular route did not produce detectable levels of serum antibody as measured by Western blot analysis. In contrast, mice given two doses of replicons followed by a single intramuscular boost with recombinant ureB produced serum antibody which reacted against ureB from pathogenic *H. pylori* in the Western blot assay. Analysis of this serum antibody to urease revealed that it was predominately IgG2a. Spleen cells from animals immunized with replicons alone or replicons plus a recombinant ureB booster immunization produced IFN-γ but not IL-4 following stimulation with recombinant ureB in vitro.

Previous studies from this laboratory have described the analysis of the immunogenicity of replicons which encode the C-fragment of tetanus toxin or gene fragments of HIV *gag* and *env* [18 and 20]. In both cases, it was found that administration of the replicons via intramuscular route resulted in the production of serum antibodies directed against the foreign protein. It was surprising then that we were unable to detect significant serum antibodies to ureB following immunization with replicons alone. We believe that an explanation for these results relies, in part, on some of the unique features of the replicon vaccine vector. Studies from our laboratory have found that expression of recombinant proteins from replicon vectors in vivo occurs rapidly post inoculation, peaking at 16–20 h and by 24–48 h post inoculation, the expression of recombinant proteins from replicons was not detected (Novak and Morrow, manuscript in preparation). Thus, the capacity of replicons alone to stimulate an antibody response might depend, in part, upon the inherent immunogenicity of the foreign protein. For some proteins, such as C-fragment of tetanus toxin, the limited expression of the antigen is sufficient to stimulate an immune response. It may be then, that the ureB protein used in this study, was not sufficiently antigenic to stimulate a serum antibody response by immunization with replicons alone. We did note that a smaller mass protein was immunoprecipitated from replicon infected cells. It is possible that this is a breakdown product from VP4-ureB protein which occurred as a result of the intracellular expression of the VP4-ureB. Since ureB would, under normal circumstances, not be expressed in the eukaryotic intracellular milieu, it is possible this protein contains cryptic proteolytic cleavage sites. The breakdown of the ureB in cells then, could have contributed to the lower immunogenicity when compared to other antigens expressed from the replicons. Further experiments will be required to resolve this question.

Although replicons alone were not sufficient to stimulate production of anti-urease antibodies, they did infect the mice and produce sufficient ureB protein to prime an immune response. This was evident from our experiments in which we detected primarily IgG2a anti-ureB antibodies in animals which had been given two doses of replicon followed by a single dose of ureB. In contrast, animals given a single dose of ureB did not have detectable serum antibodies to ureB and animals given three dosages of recombinant ureB produced a predominantly IgG1 antibody response. The prevalence of an IgG2a antibody response in the replicon immunized animals is consistent with the stimulation of a Th1 helper response [28]. To confirm this, we analyzed the spleen cells from animals immunized with replicons plus a single dose of recombinant ureB for the capacity to produce specific cytokines upon stimulation with recombinant ureB. We detected production of IFN-γ but little or no IL-4 from ureB-stimulated spleen cells obtained

from animals given only replicons. Moreover, even in mice when no detectable antibodies were seen (Fig. 2, group C) IFN-γ was detected in splenic T cells cultures (Fig. 4, columns 3). Thus, even though we did not detect humoral response, we were able to show that a cellular immune response had been stimulated by replicons encoding ureB. The ability of the replicons to stimulate a Th1 type response is generally consistent with results obtained from other vectors where the expression of foreign protein occurred intracellularly. Most interestingly, immunization with plasmid DNA has been shown to stimulate primarily a Th1 response [26]. The immune response observed following DNA immunization parallels that seen with replicons in that both vector systems produce low levels of antigen which many times does not result in a vigorous antibody response [22].

Previous studies have reported on the murine CD4 T cell responses from mice immunized with sonicates from the closely related *Helicobacter felis* [16 and 17]. The cellular response to the immunization resulted in a primarily Th1 type response to re-challenge and the cellular proliferation assay correlated with the severity of gastric inflammation, suggesting that the Th1 type response promoted a local delayed type hypersensitivity response in the animals. Based on the results of these studies, it was concluded that the Th1 cells contributed to the pathogenesis of the disease [16, 17 and 27]. Just what stimulated these Th1 cells to become pathogenic was not clear since the antigen within the *H. felis* lysate which stimulated the response was not identified. A comparison of the immune response and gastric inflammation as a result of any vaccine approach is then important. Unfortunately, PVR 1 mice are not susceptible to orally administered poliovirus and we are unable to determine whether oral administration with replicons will induce mucosal antibodies in mice. However, having shown that ureB replicons are immunogenic in mice given via the intramuscular route, it might be possible to combine parenteral immunization using replicons with oral immunization with recombinant ureB. A previous study using influenza virus found that systemic immunization followed by oral administration of antigen resulted in protective antibodies in the secretions [18]. As a general vaccine strategy though, this approach is limited because most of the antigens are unstable at the low pH environment of the intestine. Given the fact that *H. pylori* is adapted to the gastrointestinal tract, the ureB protein which is located on the surface of the bacteria, is probably more stable than most antigens in this environment. Experiments are underway to test combinations of systemic priming with oral administration of ureB for immunogenicity. Recently we have established a mouse challenge model for *H. pylori* infection [27]. In future experiments we will use this model to determine whether the immune response following immunization with ureB replicons confers protection to challenge with *H. pylori.* It might also be possible to extend the characterization of the replicons to vaccinate non-human primates orally with the vector and test for the presence of mucosal anti-urease antibodies, since the administration of oral poliovirus vaccine to non-human primates is known to stimulate both systemic and mucosal antibodies [24].

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Protection of turkeys against *Chlamydia psittaci* challenge by gene gun-based DNA immunizations

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Abstract

Particle-mediated (Helios® Gene Gun) transfer to the turkey epidermis of plasmid DNA expressing the major outer membrane protein (MOMP) of an avian *Chlamydia psittaci* strain was evaluated for its ability to raise an immune response and protection against challenge with the homologous strain. In turkeys, the delivery of pcDNA1/MOMP coated onto 0.6 μm gold beads was the most efficient compared to immunisations using 1.0 or 1.6 μm gold beads. The delivery of as little as 1 μg pcDNA1/MOMP coated onto 0.6 μm gold beads was efficient. Immunisation with 1.0 μm gold beads required twice more (2 μg) DNA to achieve comparable results. The use of 2 μg DNA coated onto 1.6 μm gold beads had no effects. The gene gun delivery both primed T-helper and B-cell memory although recombinant MOMP-expressing cells did not induce high-titre antibody responses. The significance of

gene gun-based DNA immunisation as a means of preventing severe clinical signs, lesions and chlamydia excretion in a turkey model of *Chlamydia psittaci* infection was demonstrated.

Author Keywords: DNA vaccine; Chlamydia; Turkeys

Article Outline

1. Introduction

Observations in the early 1990s that plasmid DNA could directly transfect animal cell in vivo resulted in the exploration of the use of DNA plasmids to induce protective antibody and cell-mediated immune responses by direct injection into animals of DNA encoding antigenic proteins. Nowadays, DNA immunisation has been used to elicit protective immune responses in a variety of animal models for parasitic, viral and bacterial diseases including *Chlamydia trachomatis* and *Chlamydia psittaci* [1, 2, 3, 4, 5, 6, 7, 8, 9 and 10].

The only protective chlamydial antigen which has been unambiguously identified is the major outer membrane protein (MOMP). This protein, represents the majority of the surface exposed protein of *C. psittaci*. It is a protein of approximately 40 kDa characterised by four variable regions and five intervening constant regions of conserved structure and function. MOMP is an immunodominant protein carrying genus-, species- and interestingly serovar-specific epitopes eliciting neutralising antibodies [11, 12 and 13].

Several methods for the delivery of DNA vaccines have been evaluated. Parenteral routes of inoculation that achieved good protection included intramuscular and intravenous injections. Successful mucosal routes of vaccination included DNA drops administered to the nares or trachea. In addition, gene gun

delivery of DNA into the epidermis seems to be a very efficient method of inoculation, achieving protection with 250 to 2500 times less DNA than direct inoculations of purified DNA in saline [14]. Gene gun delivery takes advantage of the ability of balistically accelerated microscopic gold particles to penetrate cell membranes without killing the cell. By mixing these gold beads with purified plasmid DNA in the presence of polycations, the DNA becomes coated onto the gold particles.

In the present study the Helios® gene gun (BioRad) was evaluated for achieving protective immunological responses in turkeys against *C. psittaci* challenge. The effects of gold bead size on the outcome of immunological and protective responses are evaluated.

2. Materials and methods

2.1. *C. psittaci* strain

In this study, *C. psittaci* strain 84/55, isolated from the lungs of a diseased parakeet, was used. The strain was previously characterised using both serovar-specific monoclonal antibodies and restriction fragment length analysis of the *omp1* gene. Strain 84/55 was classified as an avian serovar A and genotype A strain [15 and 16]. The strain was grown in Buffalo Green Monkey (BGM) cells as previously described [17] and the 50% tissue culture infective dose ($TCID_{50}$) was determined on BGM cells by the method of Spearman and Kärber [18].

2.2. Vaccine DNA

Plasmid pcDNA1/MOMP was constructed by sticky-end ligation of the outer membrane protein 1 (*omp 1*) gene of strain 84/55 into the *Eco*RI site of pcDNA1 [19 and 20]. DNA's were grown in *Escherichia coli* MC1061/P3 bacteria and purified by use of the QIAGEN Tip 2500 plasmid preparation method (QIAGEN GmbH, Hilden, Germany). DNA concentration was determined by optical density at 260 nm and confirmed by comparing intensities of ethidium bromide stained *Eco*RI restriction endonuclease fragments with standards of known concentration. DNA was stored at -20°C in 1 mM Tris (pH 7.8), 0.1 mM EDTA. Expression of MOMP was confirmed by indirect imunofluorescent staining of both DEAE dextran transfected COS7 cells and turkey skeletal muscle injected with pcDNA1/MOMP [16]. For gene gun inoculations, 2 μg pcDNA1/MOMP diluted in saline (0.9% NaCl) was coated onto 1 mg gold beads of either 0.6, 1.0 or 1.6 μm. DNA coating was performed as described by the manufacturer. pcDNA1 was used as control plasmid.

2.3. Vaccination trial

SPF turkeys (CNEVA, Ploufragan, France) were divided into eight groups, reared in negative pressure isolators on wired floors. Turkeys of groups 1 to 6 (n=5) were immunised by DNA-coated gold particles delivered to a shaved and ethanol disinfected area of the back skin by use of the Helios® Gene Gun (Bio-Rad). DNA-coated gold beads were accelerated into the epidermis using a helium pressure setting of 200 p.s.i. Turkeys of groups 1–3 received one shot of 0.5 mg gold beads (1 μg DNA/shot) of respectively 0.6, 1.0 or 1.6 μm diameter. Turkeys of groups 4–6 received two shots (1 μg DNA/shot) of 0.6, 1.0 or 1.6 μm diameter, respectively. Group 1–6 received a first gene gun inoculation at time 0 and the second 3 weeks later. *C. psittaci* serovar A challenge was administered by aerosol at 14 days after the second DNA inoculation (35 days PPV). The challenge consisted of $10^{8.5}$ $TCID_{50}$ of chlamydia strain 84/55. A control group (group 7) (placebo-vaccinated-challenged group) of six pcDNA1 vaccinated SPF turkeys was similarly infected. A second control group (group 8) of five pcDNA1 vaccinated SPF

turkeys remained unchallenged. The latter two groups had been injected with 2.0 μg pcDNA1, precipitated onto 1.0 μm gold particles.

2.4. Samples

All turkeys were daily observed for clinical signs. Nasal and cloacal swabs were taken every other day. Blood samples were collected for the detection of anti-MOMP specific antibodies immediately prior to each DNA inoculation, immediately prior to the experimental infection and at 8 and 18 days after the challenge infection. Blood samples were stored overnight at room temperature, centrifuged (325×*g*, 10 min, 4°C) and afterwards serum was collected and frozen at -20°C until tested. At the time of euthanasia, 18 days postchallenge, proliferative responses in peripheral blood lymphocytes were examined. All euthanised turkeys were examined for macroscopic lesions. Cryostat tissue sections of the abdominal and thoracic airsacs, the lungs, the pericardium, the spleen and the liver were examined for the presence of chlamydia antigen.

2.5. Chlamydia isolation

Nasal and cloacal swabs were examined for the presence of viable chlamydiae by isolation in BGM cells, as previously described [17]. The number of BGM cells with chlamydial inclusions was counted in five ad random selected microscopic fields (500×). For each individual turkey, the excretion was ranging from score - to +++. Score - indicated no antigen present. Score +, ++ and +++ were given when a mean of 1–5, 5–10 and more than 10 inclusion positive cells per field were present, respectively. Cloacal and nasal shedding in the placebo-vaccinated control group and in pcDNA1/MOMP vaccinated groups are presented as mean scores±standard deviation in cultures of individual turkeys.

2.6. Direct immunofluorescence staining

Cryostat tissue sections were examined by the IMAGEN direct immunofluorescence staining, as previously described [17] (Novo Nordisk Diagnostics, Cambridge, UK). The number of cells with chlamydial inclusions was counted in five ad random selected microscopic fields (500×). The results are presented as a score ranging from - to +++. Score - indicated no antigen present. Score +, ++ and +++ were given when a mean of 1–5, 5–10 and more than 10 inclusion positive cells per field, respectively, were present.

2.7. Antibody responses

Enzyme-linked immunosorbent assays (ELISA's) were performed on turkey sera being pretreated with kaolin to remove background activity. Anti-MOMP antibody titres and anti-MOMP antibody isotypes were determined by ELISA as previously described [19, 20 and 21].

2.8. Lymphocyte proliferative responses

Peripheral blood leukocytes (PBL) were isolated from heparinised-blood samples obtained by venipuncture from each turkey of groups 1–8, at 18 days after challenge. Macrophage-like adherent cells were removed from cell suspensions by treatment of heparinised-blood with carbonyl iron powder as described by Sjöberg et al. [22]. Subsequently, turkey PBL were isolated over a lymphoprep density gradient (Life Technologies) and most remaining macrophages were removed by adherence to plasma-coated polystyrene culture plates. PBL responses to chlamydia rMOMP were measured as previously described [21].

2.9. Statistics

The two-tailed Student *t*-test was employed for all statistical analyses. Results were considered significantly different at the level of $P<0.05$.

3. Results

3.1. Protection against *C. psittaci* challenge

Following two inoculations with pcDNA1/MOMP or pcDNA1, SPF turkeys were challenged with a very high experimental infective dose of the homologous chlamydia strain. Severe clinical signs and lesions were only observed in placebo-vaccinated challenged control turkeys (group 7) and in groups 3 and 6, both vaccinated with pcDNA1/MOMP coated onto 1.6 μm gold particles. All turkeys in these three groups showed depression, anorexia, conjunctivitis, head shaking, respiratory distress and diarrhoea. At necropsy, conjunctivitis, rhinitis, sinusitis, pneumonia, diffuse fibrinous airsacculitis, sero-fibrinous to fibrinous adhesive pericarditis, hepatosplenomegaly and congestion of the kidneys were observed. In two out of five turkeys vaccinated with 1.0 μg pcDNA1/MOMP coated onto 1.0 μm gold particles (group 2), only conjunctivitis, head shaking and respiratory distress were observed. Necropsy findings in these two turkeys included conjunctivitis, rhinitis, sinusitis, pneumonia, bilateral focal fibrinous airsacculitis, hepatosplenomegaly and congestion of the kidneys. The three remaining turkeys of group 2 showed only head shaking and revealed only one or two small fibrin cloths unilaterally in the abdominal airsacs. Turkeys vaccinated with 1 or 2 μg DNA coated onto 0.6 μm gold particles (groups 1 and 4, respectively) and turkeys vaccinated with 2.0 μg DNA coated onto 1.0 μm gold particles (group 5) showed no clinical signs. The abdominal airsacs of all these turkeys revealed unilaterally one or two small fibrin cloths. No further lesions were observed in these three groups.

Results of the cloacal or nasal cultures obtained at two-daily intervals during 18 days following challenge are shown in Table 1 and Table 2, respectively. A significant level of protection was observed in all turkeys of groups 1, 4 and 5. Turkeys of these three groups showed no cloacal shedding during the 18 days of observation. All placebo-vaccinated challenged control turkeys (group 7), all turkeys of groups 3 and 6, and 40% (2 of 5) of the turkeys of group 2 had positive cloacal cultures during the 18 days of observation. All control turkeys as well as all vaccinated turkeys excreted chlamydia nasally. However, the period of shedding was significantly shorter in turkeys of groups 1, 4 and 5. In the latter groups, nasal excretion was no longer observed than 6 days postchallenge, while at 18 days postchallenge 66% (4 of 6) of the control turkeys, 40% (2 of 5) of the turkeys of group 2 and 60% (3 of 5) of the turkeys of groups 3 and 6, still excreted chlamydia nasally. All the turkeys in the sham-immunised, nonchallenged group remained chlamydia negative throughout the experiment.

Table 1. Results of cloacal cultures of placebo-vaccinated controls and of pcDNA1/MOMP vaccinated turkeys after challenge

 (21K)

Table 2. Results of nasal cultures of placebo-vaccinated controls and of pcDNA1/MOMP vaccinated turkeys after challenge

 (23K)

Immunofluorescence staining of tissues of the control turkeys (group 7), euthanised 18 days postchallenge revealed strong chlamydia replication in air sacs, lungs and liver and weak replication in pericardium and spleen (Table 3 and Table 4). Regarding chlamydia replication, there were significant differences in protection provided by the different gene gun-based immunisation methods. The best protection occurred in groups 1, 4 and 5.

Table 3. Chlamydia replication in tissues of placebo-vaccinated turkeys and turkeys vaccinated with 1 μg pcDNA1/MOMP, 18 days postchallenge determined by direct immunofluorescence staining. - means no antigen present, + an average of 1–5 inclusion positive cells, ++ an average of 5–10 inclusion positive cells and +++ an average of more than 10 inclusion positive cells

(9K)

Table 4. Chlamydia replication in tissues of turkeys vaccinated with 2μg pcDNA1/MOMP, 18 days postchallenge determined by direct immunofluorescence staining. - means no antigen present, + an average of 1–5 inclusion positive cells, ++ an average of 5–10 inclusion positive cells and +++ an average of more than 10 inclusion positive cells

(9K)

3.2. Antibody responses

Three weeks following the first immunisation with pcDNA1/MOMP anti-MOMP serum antibodies were observed in all turkeys of group 5, and in 2 of 5 turkeys of groups 1, 2 and 4 (Table 5 and Table 6). Following the second immunisation anti-MOMP antibodies also appeared in the three remaining seronegative turkeys of groups 1, 2 and 4. At that time, anti-MOMP antibody titres were not detected in the turkeys immunised by use of 1.6 μm gold beads. The antibody responses as determined in an ELISA with homologous rMOMP were however weak. Following challenge, all control turkeys, as well as all turkeys of groups 3 and 6, displayed an antibody response. Protection, as determined by chlamydia isolation from cloacal and nasal swabs and by antigen detection in tissues, occurred in all vaccinated turkeys, except for 2 turkeys of group 2. However, the best protection occurred in turkeys which had not displayed a secondary antibody response upon challenge. All placebo-vaccinated noninfected turkeys of group 8 showed undetectable anti-MOMP antibody activity in the lowest serum dilution (1/32) used.

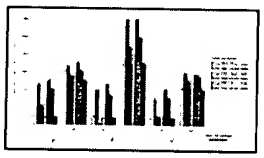
Table 5. MOMP-specific antibody titres following DNA vaccination (1μg DNA) and subsequent challenge with the homologous *C. psittaci* strain. Prevac, bleed at haching, before DNA vaccination; PPV, postprimovaccination; PBV, postboostervaccination; PC, postchallenge; < no antibodies detected at used serum dilution (1/32)

(6K)

Table 6. MOMP-specific antibody titres following DNA vaccination (2 μg DNA) and subsequent challenge with the homologous *C. psittaci* strain. Prevac, bleed at haching, before DNA vaccination; PPV, postprimovaccination; PBV, postboostervaccination; PC, postchallenge; < no antibodies detected at used serum dilution (1/32)

(13K)

The gene gun immunisation method induced low IgM, IgG and IgA responses (Fig. 1). Indeed, 14 days after the second DNA inoculation (35 days PPV), all vaccinated turkeys of groups 1, 4 and 5 and 3 on 5 turkeys of group 2 showed for all isotypes low mean O.D. values of 0.2 to 0.3, respectively, above those of sera of turkeys immunised with the control plasmid. Eighteen days after challenge (53 days PPV), these marginal IgM, IgG and IgA levels had increased, with IgG dominating the immune response. However, increased levels of IgG were not significantly correlated with higher levels of protection. In placebo-vaccinated turkeys the IgM, IgG and IgA antibody levels following infection were identical to those of immunised groups 3 and 6, i.e. the groups with no protection (Fig. 1).



(35K)

Fig. 1.

3.3. Antigen-specific lymphocyte proliferation

Proliferative responses to rMOMP of peripheral blood lymphocytes (PBL) of pcDNA1/MOMP or pcDNA1 immunised control turkeys were determined 18 days following the challenge with the homologous chlamydia strain. The PBL of control turkeys (group 7) and of pcDNA1/MOMP immunised turkeys of groups 3 and 6, displayed comparable low proliferative responses. All other immunised turkeys displayed significant higher proliferative responses (Table 7) when compared to the PBL of the controls, with similar proliferative responses in groups with the highest degree of protection (groups 1, 4 and 5). The PBL responses of challenged controls were comparable to responses of nonchallenged controls.

Table 7. Proliferative response of peripheral blood lymphocytes of immunized or nonimmunized turkeys to rMOMP day 18 postchallenge

(9K)

4. Discussion

In the present study, the eukaryotic expression vector pcDNA1/MOMP, encoding the major outer membrane protein of an avian *C. psittaci* serovar A strain, was used to asses the potential of DNA-mediated immunisation of turkeys against challenge with the homologous chlamydia strain. The efficacy of gene gun delivery of plasmid DNA-coated gold particles to the skin was evaluated. The gene gun vaccination method reflects efficient biolistic transfection together with efficient antigen presentation by cells of the birds skin associated lymphoid tissue (Langerhans cells).

A significant level of protection was only achieved in turkeys immunised with 1 or 2 μg plasmid DNA coated onto 0.6 μm gold beads (groups 1 and 4) and in turkeys immunised with 2 μg plasmid DNA coated onto 1.0 μm gold beads (group 5). However, in these three groups, chlamydiae could still replicate in epithelial cells and macrophages of the respiratory tract, resulting in positive nasal culture for 6 days after challenge. Possibly, the protection level at the respiratory tract would have been higher if we had chosen a less severe challenge. Notwithstanding the severe challenge used, replication outside the respiratory tract was only observed in 1 out of 15 turkeys of groups 1, 4 and 5. By contrast, in all turkeys vaccinated by use of 1.6 μm gold beads (groups 3 and 6), and in all control turkeys, chlamydiae replicated intensively outside the respiratory tract, resulting in positive cloacal cultures throughout the observation period. Chlamydia replication in the pericardium of 1 turkey vaccinated by use of 0.6 μm gold beads (group 4) might be due to the close contact with the surrounding infected airsacs, rather than by septicaemia, as replication was not observed in liver nor spleen and because all cloacal cultures remained negative throughout the observation period.

Gene gun delivery of pcDNA1/MOMP primed both T-helper and B-cell memory although rMOMP-expressing cells did not induce high-titre antibody responses. Evidence for the mobilisation of B-cell memory in response to challenge was found in gene gun vaccinated turkeys of groups 1, 4 and 5, as IgG levels where significantly higher than IgM levels. Evidence for the mobilisation of T-cell memory in response to challenge was found in all turkeys vaccinated by use of 0.6 or 1.0 μm gold beads as shown by the significantly increased PBL proliferative responses following challenge when compared to placebo-vaccinated control turkeys and to groups 3 and 6.

Gene gun delivery of DNA coated on 0.6 or 1.0 μm gold beads to the birds skin was a very efficient immunisation method. The use of 1.6 μm gold beads had no effects. The smaller the beads, the more beads/mg. Therefore, when small beads are used more cells can become transfected. Furthermore, the large beads probably penetrate into the deeper dermal layer, while the small beads were accelerated into the epidermal layer being rich in antigen presenting cells. In the present study, protection levels correlated well with antibody levels and antigen-specific cell proliferation. This is at apparent similarity with earlier studies comparing different DNA inoculation methods [14]. Therefore, the Helios® Gene Gun system obviously represents a very efficient transfection method. When the birds skin is transfected, DNA expressing antigens become most likely subject to immune surveillance by Langerhans cells present in the birds skin. Like in mammals these act as antigen presenting cells (dendritic cells) that are capable of presenting DNA expressed antigens to the spleen, where antigen-loaded antigen-presenting cells can activate naive T cells.

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